The Role of Helper NB-LRRs in Late Blight resistance

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Table of Contents

Abstract

Late blight is economically the most important disease in potato. A novel approach to produce resistant cultivars is the introduction of *R* genes into cultivars by using cisgenesis. The success of this approach depends on the background of the receiving cultivars. In a previous project, transformation with Rpi-chc1 led to resistance in Desiree, but not in Premiere. One explanation for this could be a difference in the presence or absence of 'helper' NB-LRRs. Previous research has shown that several *R* genes require one or more additional 'helper' NB-LRRs to activate defence signalling and trigger a HR. In tomato, potato and *N. benthamiana*, one class of 'helper' NB-LRRs, the NRC genes, has been shown to be required for several *R* genes including Rpi-chc1. In this study, to explain the background dependent success of introducing *R* genes into potato, the requirement and diversity of NRCs in potato and *N. benthamiana* was studied. This was done by mining for allelic variants of NRCs in potato and by performing Virus Induced Gene Silencing (VIGS) assays and overexpression studies in *N. benthamiana*. This study shows that different NRCs show different levels of conservedness. Potato, tobacco and *N. benthamiana* appear to have different numbers of NRC1, NRC2 and NRC3 genes, indicating a different diversification of these genes in these species. The evidence that suggests that Rpi-chc1 requires NRCs to trigger a HR could not be reproduced. However, this study does demonstrate that Rpi-ber might require tobacco NRC3 to trigger a full HR, and that overexpression of tomato NRC3 or potato NRC3 in *N. benthamiana* reduces the HR triggered by Rpi-ber. The results from this study highlight that it is worth further investigating the diversity of potato NRCs and their importance in *R* gene triggered resistance.

1. Introduction

Late blight, caused by *Phytophtora infestans*, is economically the most important disease in potato. This rapidly adapting pathogen can destroy a potato crop within a few days when left unchecked (Fry, 2008). Already in the 19th century, late blight was a threat to potato production, as became very clear during the Irish potato famine. In the last century, a lot of research has been done to learn more about this disease, the defence repertoire that plants use to defend themselves, and how breeders can create more resistant varieties. Plant defence is activated after recognition of the pathogen by plant receptors. Plants contain both extracellular and intracellular receptors that can recognise pathogen molecules. Until recently, it was commonly accepted that extracellular pattern recognition receptors (PRRs) recognise pathogens by detecting conserved microbial molecules, also known as pathogen associated molecular patterns (PAMPs) (Bent and Mackey, 2007); and that intracellular receptors known as Resistance proteins or *R* genes, detect effector proteins that the pathogen produces to manipulate its host (Dodds and Rathjen, 2010). However, several studies now show that the distinction between PAMPs and effectors, and PRRs and *R* genes is not as strict as previously thought, as not all microbial defence activators can be grouped as either PAMPs or effectors (Thomma et al., 2011). The most common R proteins are the NB-LRR proteins (nucleotide binding site, leucin rich repeat). Recognition of effectors by an NB-LRR protein leads to a strong defence response, which includes programmed cell death, known as the hypersensitive response (HR) (Vleeshouwers et al., 2011). When an effector is recognised by an NB-LRR protein, it is referred to as an avirulence protein (AVR). A common model to describe the interaction between NB-LRR proteins and Avr-proteins is the gene-for-gene model. This gene-for-gene model states that a one-on-one relationship exists between an AVR protein and a NB-LRR protein, where a NB-LRR specifically recognises the presence or activity of a specific AVR-protein. R proteins can recognise an AVR protein both directly or indirectly (Jones and Dangl, 2006). In the case of indirect recognition, the R protein recognises changes in a third protein in the plant that are induced by the AVR protein. This third protein can be the operational target of the AVR protein, or it can be a mimic of the operational target. These targets are referred to as "guardee" or a "decoy", respectively (van der Hoorn and Kamoun, 2008; Cesari et al., 2014).

Although most NB-LRR receptors function fully on their own, several NB-LRR receptors require additional NB-LRR 'helper' proteins to confer recognition of- and successive resistance against pathogens (Sinapidou et al., 2004; Eitas and Dangl, 2010; Cesari et al., 2014; Wu et al., 2015a; Wu et al., 2015b). One of the first found examples of this is the RPP2A/RPP2B pair in *Arabidopsis thaliana*. It was found that both *RPP2A* and *RPP2B* are required to confer resistance against *Hyaloperonospora arabidopsidis* isolate Cala2 (Sinapidou et al., 2004). Several more paired NB-LRRs have now been identified. Recent studies on the NB-LLR pairs RRS1/RPS4 in *A. thaliana* and RGA4/RGA5 in rice have given us a new insight in how these pairs might operate. In both cases, the NB-LRR proteins form homo- and hetero complexes, and in both cases each member of the pair achieves one specific task; one, the sensor NB-LRR, is involved in AVR-recognition and the other, the helper NB-LRR, in downstream signalling (Cesari et al., 2014). In plant breeding, knowledge about NB-LRR pairs can be very valuable, as both members of the NB-LRR pairs are required for functional resistance. Although still relatively little is known about these helper NB-LLRs, NB-LRR pairs have now been identified in several plant crop species, including *Arabidopsis*, Tobacco, Wheat and Rice (Eitas and Dangl, 2010).

In potato resistance breeding, one focus is the identification of new *R* genes against *P. infestans* in potato and its wild relatives. Several of these *R* genes have now been crossed into commercial cultivars. Unfortunately, because *P. infestans* is a rapidly evolving pathogen, it can overcome recognition by single *R* genes relatively quickly (Fry, 2008). To increase the durability of resistance against *P. infestans*, one approach is therefore to stack multiple *R* genes in plants.

This makes it more difficult for a pathogen to overcome recognition, because a change in one of its effector proteins is no longer sufficient to avoid recognition. Several potato genotypes that contain stacks of multiple *R* genes already exist. Unfortunately, *R* gene stacking in potato by genetic crossings is not ideal. Because of the high heterozygosity in potato, desired characteristics will never be fully recovered after genetic crosses. An alternative is therefore to use cisgenesis to stack R-genes in potato cultivars (Jo et al., 2014). For cisgenic stacking in potato, knowledge about potential NB-LRR helper proteins can be very valuable. If only one member of a NB-LRR pair is introduced in a potato genotype, one could expect that the newly introduced resistance will be suboptimal or even absent when the other required NB-LRR protein is missing. The WUR Laboratory of Plant Breeding has introduced the late blight resistance gene *Rpi-chc1* in potato cultivars Desiree and Premiere. In Desiree, this led to resistance. In Premiere, however, transformation with *Rpi-chc1* did not lead to resistance. This raises the question why the introduction of an *R* gene is successful in one genotype, but not in another. One hypothetical answer here could be that Rpi-chc1 cooperates with a NB-LRR that is present in Desiree, but not in Premiere.

Relatively little is still known about the presence and diversity of helper NB-LRRs in *Solanum* species. Recent research has however suggested that in *Solanum* species, several *R* genes require helper NB-LRRs to induce HR (Wu et al., 2015a; Wu et al., 2015b, Laboratory of Plant Breeding; Unpublished data). Already in 2007, a study by (Gabriëls et al., 2007) demonstrated that a CC-NB-LRR termed Sl-NRC1, is required for Cf-4-mediated HR and resistance in tomato. A VIGS (virus induced gene silencing) screen with *R* genes in *N. benthamiana* furthermore hypothesised that Nb-NRC1 (the *N. benthamiana* homolog of NRC1) is required for the HR triggered by multiple other R-genes in in *N. benthamiana.* (Gabriëls et al., 2007). Interestingly, NRC1 most likely doesn't exist in the *N. benthamiana* genome. Instead of NRC1, several homologs of Sl-NRC1 are present in the genome of *N. benthamiana*; Nb-NRC2a, Nb-NRC2b, Nb-NRC2c and Nb-NRC3 (Wu et al., 2015b). A physical association between these Nb-NRCs and R-proteins still needs to be demonstrated. However, another NB-LRR 'helper' gene, NRB1, was identified in *N. benthamiana*, whose product physically associates with the *Solanum bulbocastanum* receptor Rpi-blb2. NRB1 was shown to be required for Rpi-blb2 mediated HR and late blight resistance(Wu et al., 2015a). Furthermore, overexpressing NRB1 led to an enhanced Rpi-blb2-mediated HR induced by AVRblb2 homologs that normally only induce a weak or no HR.

			NRC4	NRC2a	NRC3	NRC2c	
	EV	**NRB1**	**NRC-4611**	**NRC-26706**	**NRC-11087**	**NRC-31134**	**SGT1**
Rpiblb2	HR	No HR	HR	HR	HR	HR	
Mi	HR	No HR	HR	HR	HR		
R1	HR	No HR	HR	HR	HR		
Prf	HR	HR	HR	No HR	Reduced HR	Reduced HR	
Sw5	HR	HR	HR	Reduced HR	No HR		
Rx	HR	HR	HR	HR	HR	HR	
Bs2	HR	HR	HR	HR	HR	HR	
Cf4	HR	HR	HR	HR	HR	HR	
R8	HR	HR	HR	HR	HR	HR	No HR
Rx	HR	HR	HR	HR	HR	HR	
Gpa2	HR	HR	reduced HR	No HR	No HR	Reduced HR	No HR
Cf9	HR		HR	HR	HR	HR	reduced HR
INF1	HR	HR	HR	HR	HR		
vnt1	HR	HR	HR	HR	HR	HR	reduced HR
chc1	HR	HR	reduced HR	No HR	HR	No HR	No HR
chc2	HR	HR	HR	HR	HR	HR	No HR
R3a	HR	HR	HR	HR	HR	HR	No HR
R3b	HR	HR	HR	HR	HR	HR	No HR
sto1	HR	HR	HR	HR	HR	HR	No HR
blb3	HR		HR	HR	HR	HR	No HR
R9a	HR	HR	HR	HR	HR	HR	No HR

Figure 1: VIGS Screens for R-genes requiring helper NB-LRR genes. In the first column, the names of the R-genes used in the screen are shown. The NB-LRRs used in the screen are NRB1, NRC-4611 (NRC4), NRC-26706 (NRC2a), NRC-11087 (NRC3), NRC-31134 (NRC2c). EV is a negative control where no HR is expected, STG1 a positive control where HR is expected. Green cells represent data from Chih-Hang Wu of the Sainsbury Laboratory; blue data represent data from Gert van Arkel of the WUR Laboratory of Plant Breeding.

In order to map the diversity of the requirements of NB-LRRs by potato *R* genes, the WUR laboratory of Plant Breeding has performed a VIGS (Virus-induced gene silencing) screen where several Nb-NRCs were silenced (Fig. 1). It appears that *R* genes don't all require the same Nb-NRCs. Interestingly; closely related *R* genes don't always require the same Nb-NRCs to trigger HR. The potato R-genes Rpi-chc1 and Rpi-chc2 show a very different response to silencing of individual Nb-NRCs (Fig. 1), while these NB-LRRs are very similar in protein sequence (Fig. 2).

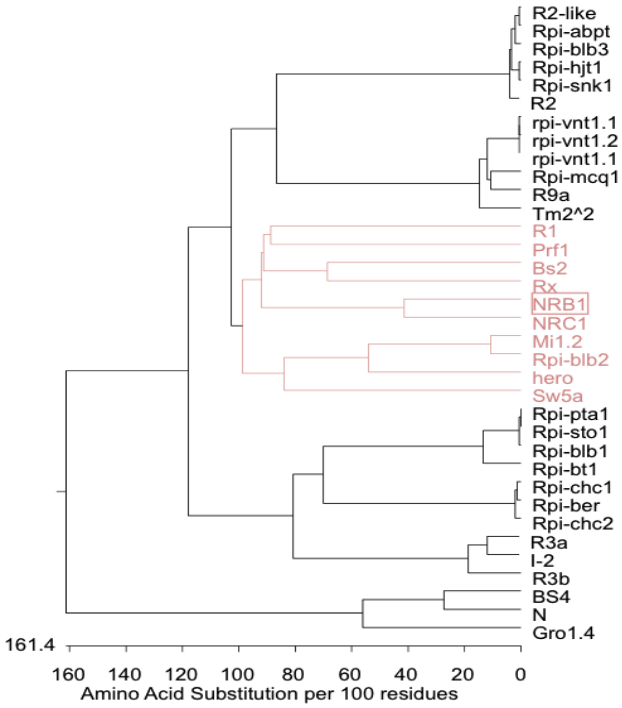


Figure 2: The phylogeny of NRB1 and *Solanaceae* R-genes

The observation that Rpi-chc1 requires NRC2a, NRC2c and NRC4 to trigger a HR raises the question whether Premiere and Desiree might not have these NRCs, or whether Premiere does not have these NRC genes. If so, it could explain the differences in success of introducing Rpi-chc1 in these backgrounds. Furthermore, it raises the question whether the closely related R-genes Rpi-ber and Rpi-tar also require NRCs to trigger a HR. In this minor thesis project, the diversity of NRCs and alleles in potato were studied. A VIGS screen was performed to map the NRCs that are required for Rpi-chc1, Rpi-chc2, Rpi-tar and Rpi-ber to trigger a HR. An overexpression assay was performed to test the effect of overexpressing NRCs on the HR triggered by Rpi-chc1, Rpi-chc2, Rpi-tar and Rpi-ber. This study demonstrates that the diversity and conservedness of NRC genes differs greatly between the different NRC genes, and that different species show a different diversification of NRC genes. Secondly, a first indication was found that Rpi-ber might require NRC3 to trigger HR. Third; this study demonstrates that the overexpression of helper NB-LRRs can inhibit the hypersensitive response triggered by *R*-genes.

2. Results

Allele mining and cloning of NRCs in potato

To find homologs of tomato NRCs in potato, a BLAST was performed with Sl-NRC1, similarly as done by (Wu et al., 2015b). 5 NRC homologs in potato were found (Table 1, Fig. 3). Protein sequences of the potato NRCs (St-NRCs) were aligned together with the NRC sequences from tomato (Sl-NRCs) and *Nicotiana benthamiana* (Nb-NRCs). Based on this alignment, St-NRC1a and St-NRC1 clustered together with Sl-NRC1; St-NRC2 clustered with Sl-NRC2 and both St-NRc3a and St-NRC3 clustered with Sl-NRC3 (Fig. 3). Interestingly, the different species differ in the amount of NRCs they have. Tomato has one version of Sl-NRC1, Sl-NRC2 and Sl-NRC3. Tobacco on the other hand, does not have a NRC1, but has three versions of NRC2. Potato has 1 version of NRC1, 1 version of NRC2 and 2 versions of NRC3; St-NRC1a could not be verified *in vivo,* as described further below.

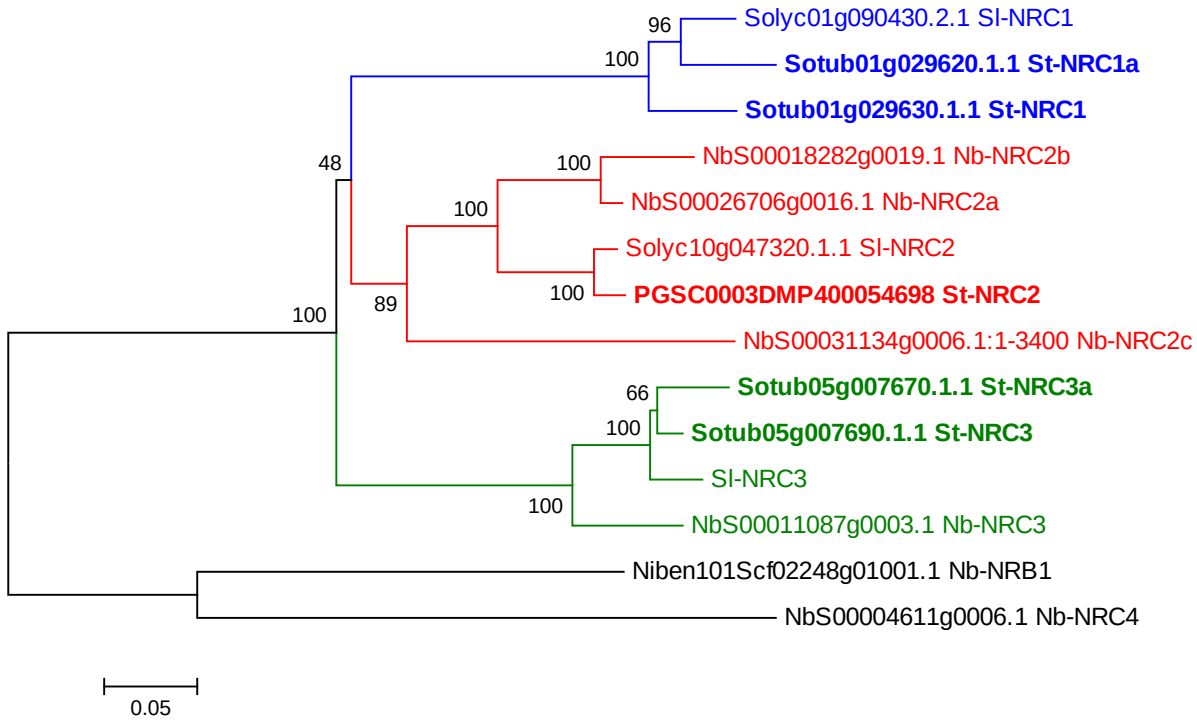


Figure 3. Phylogenetic relationship based on the protein sequences of NRCs from tomato, potato and *N. benthamiana* and Nb-NRB1. Values represent bootstrap support with 1000 replicates. The scale represents evolutionary distance (substitutions per position). The NRC1 cluster is coloured blue, NRC2 cluster is coloured red, and the NRC3 cluster is coloured green. Sequences of Nb-NRB1 and Nb-NRC4 were also included. These sequences were not identified in the allele mining study, but Nb-NRC4 was used in the VIGS treatment described below. St-NRCs represent NRCs from potato, Sl-NRCs from tomato and Nb-NRCs from *N. benthamiana*.

By comparing the predicted coding sequence (CDS) and genomic DNA sequence of the NRC homologs, introns were identified. All genes had introns, with sizes ranging from 2.135kb to 81bp (Table 1). To test for the presence of these genes *in vivo,* a PCR was performed. Primers were designed on the beginning and the end of the coding sequence. Optimal primer annealing temperatures were determined with a 66-50°C gradient temperature PCR and a 68-50°C gradient PCR on genotype DM. PCR products were put on gel, and of St-NRC1a, no bands of the expected size were observed (Fig. S1C and S1D).

Table 1: NRC homologs in potato found in BLAST with Sl-NRC1.

Gene code	Name	Chromosome	Gene length	Predicted CDS length	No. of introns	Intron size
Sotub01g029630.1.1	StNRC1	1	4,878kb	2,715kb	2	2,135kb, 466bp
Sotub01g029620.1.1	StNRC1a	1	4,391kb	2,667kb	1	1,724kb
PGSC0003DMP400054698	StNRC2	10	3,126kb	2.712kb	1	414bp
Sotub05g007670.1.1	StNRC3a	5	4,382kb	2,562kb	3	1,239kb, 467bp, 114bp
Sotub05g007690.1.1	StNRC3	5	4,621kb	2,700kb	3	1,228kb, 81bp, 611bp

In a PCR programme optimized for fragments of up to 5kb in length, no bands at all were visible for St-NRC1a. Also with more specific primers, no bands could be produced (Fig. S2). As these primers were designed on the same genotype that was tested here *in vivo*, there is probably something wrong with the sequence of this gene in the genome database. Therefore, this gene was not used in further analyses. With the identified optimal primer annealing temperatures, a PCR was performed on DM, *S. chacoense*, Desiree and Premiere. Except for St-NRC2 in DM and St-NRC3 in *S. chacoense*, all genotype – gene combinations showed bands of expected size on gel electrophoresis. Interestingly, some differences were visible between Desiree and Premiere. Comparing the bands of St-NRC3a between Desiree and Premiere, Desiree appears to show slightly weaker bands than Premiere (Fig. 4). Furthermore, the band size of St-NRC2 appears to be a bit smaller in *S. chacoense* than in Desiree. Using a temperature gradient PCR, St-NRC2 from DM and St-NRC3 in *S. chacoense* were also amplified (Fig. S3, S4).

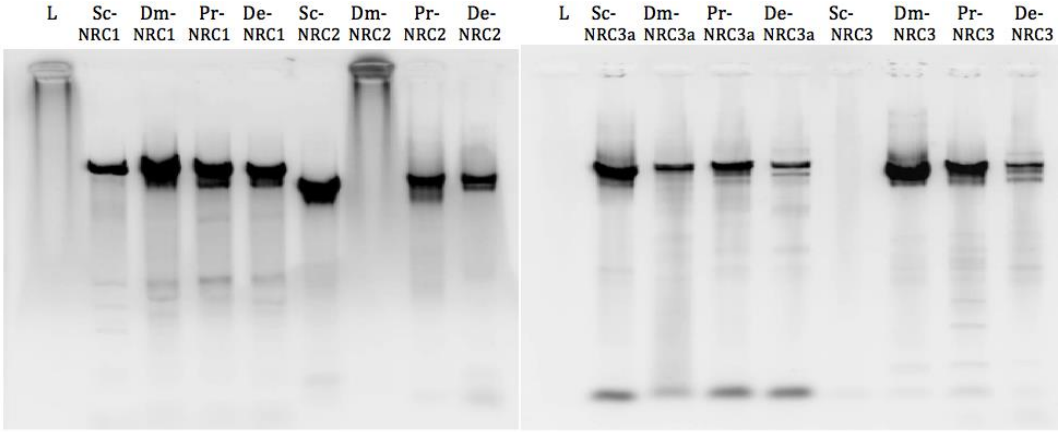


Figure 4: Gel electrophoresis of the PCR products of St-NRCs from DM (Dm), *S. chacoense* (Sc), Desiree (De) and Premiere (Pr). "L" represents the ladder.

For further sequence analysis and future cloning into expression vectors, the PCR products were cloned directly into pENTR/dTOPO, and transformed into chemically competent *E. coli* cells. As gel purification of St-NRC3 in *S. chacoense* did not yield enough DNA, and amplification with a second gradient PCR did not yield any strong bands on gel, it was not cloned into pENTR/dTOPO. To test for the presence of correct insert DNA, two colony PRCs were performed; one with the M13 forward (FW) primer that anneals in the backbone and a reverse (RV) primer that anneals to the bottom strand of the insert. A second PCR was done with M13 forward (FW) and M13 reverse (RV); these primers anneal one each side of the insert in the vector backbone. Surprisingly, when analysed with gel electrophoresis, both PCRs did not show

the expected banding patterns (300bp and 3-5kb, respectively). Eight different banding patterns were observed with M13 forward (FW) and gene specific reverse primer (Fig. 5A). Ten different banding patterns were observed with the M13 forward (FW) and M13 reverse (RV) primers (Fig. 5B).

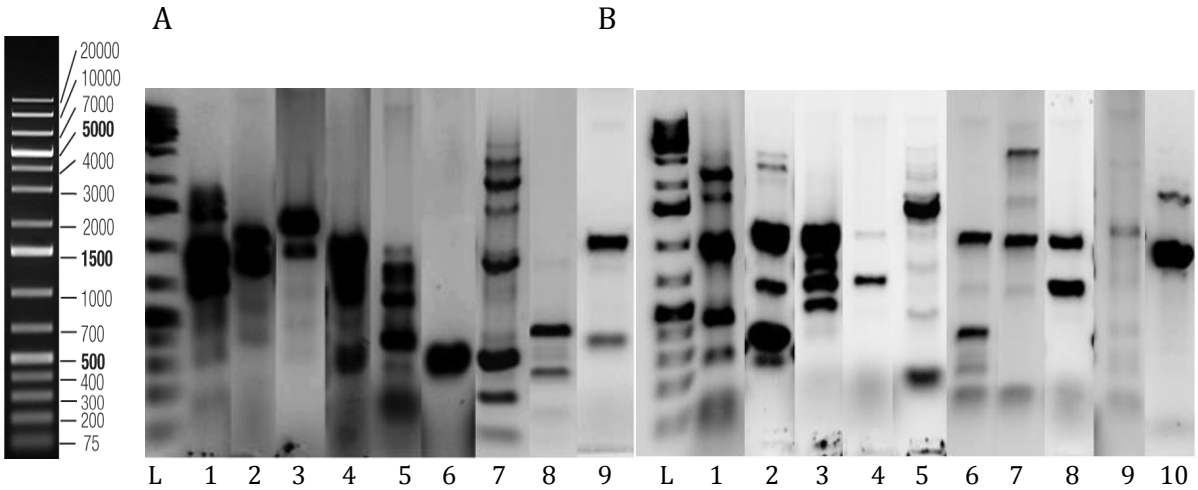


Figure 5: Different banding patterns produced by colony PCR after agarose gel electrophoresis. With M13 forward and insert reverse primer (A) and M13 forward and M13reverse (B). Numbering represents different colonies with distinct banding patterns.

Of the 327 colonies that were originally inoculated, 167 showed a banding pattern on gel. 108 showed a banding pattern after PCR with M13 forward (FW) primer and RV primer in the insert, 110 showed bands at PCR with M13 forward (FW) and M13 reverse (RV) primers. Only 51 colonies showed bands at both PCRs. Each colony that showed a distinct banding pattern was purified and sent for sequencing with the M13 forward (FW) primer. Of the colonies with genotype – NRC combinations that did not show any banding pattern, three colonies were also included. The majority of the colonies with an unexpected banding pattern had only a part of the NRC sequence inserted in the vector (Table 2). Only 15 of the colonies showed insert larger than 800bp. Further sequencing with insert specific primers showed that only 5 clones contained the complete sequences of an NRC gene, in 2 of which the insert was ligated in the opposite orientation (Table 2).

Table 2: Number of colonies that produced sequences, sequence characteristics, and banding patterns from the colonies after the colony PCR. Sequences with <-> are inserted in the vector in the inverse orientation. Numbers represent banding patterns shown in Figure 5, "X" means no banding pattern.

	Amount of sequences	**Partial insert**	**Entire insert**	**Banding pattern 300bp PCR***	**Banding pattern 5kb PCR***
Sequence produced	62	62	0		
Sequence of 1-100bp	23	23	0	X, 3, 4, 6, 7, 8	X, 1, 2, 5, 6, 7, 10
Sequence 100 – 800 bp	24	7	0	X, 1, 3, 4, 5, 6	X, 1, 6
Sequence of 800+ bp	15	9 1 (<->)	3 2 (<->)	X, 3, 4, 5, 8, 9	6, 7, X

* Numbering of banding pattern refers to Figure 5

The results of the colony PCR and sequencing were compared in order to detect the banding pattern that was associated with a complete insert. Unfortunately, no clear association could be found in the 5kb PCR. In the 300bp PCR, banding pattern 9 was unique for sequences larger than 800bp. Overall, out of the 327 colonies, only 5 yielded a complete sequence of an NRC

homolog. These 5 sequences were; 2x NRC2 from Desiree, 1x NRC2 from *S. chacoense* and 2x NRC1 from Desiree. Coding sequences were determined and translated into protein sequences for an alignment. A comparison of the two protein sequences or CDS of St-NRC1 from Desiree showed no differences between the sequences. When the protein sequence of NRC1 from Desiree was aligned with the protein sequence of DM, 68 differences showed up (Fig. 6).

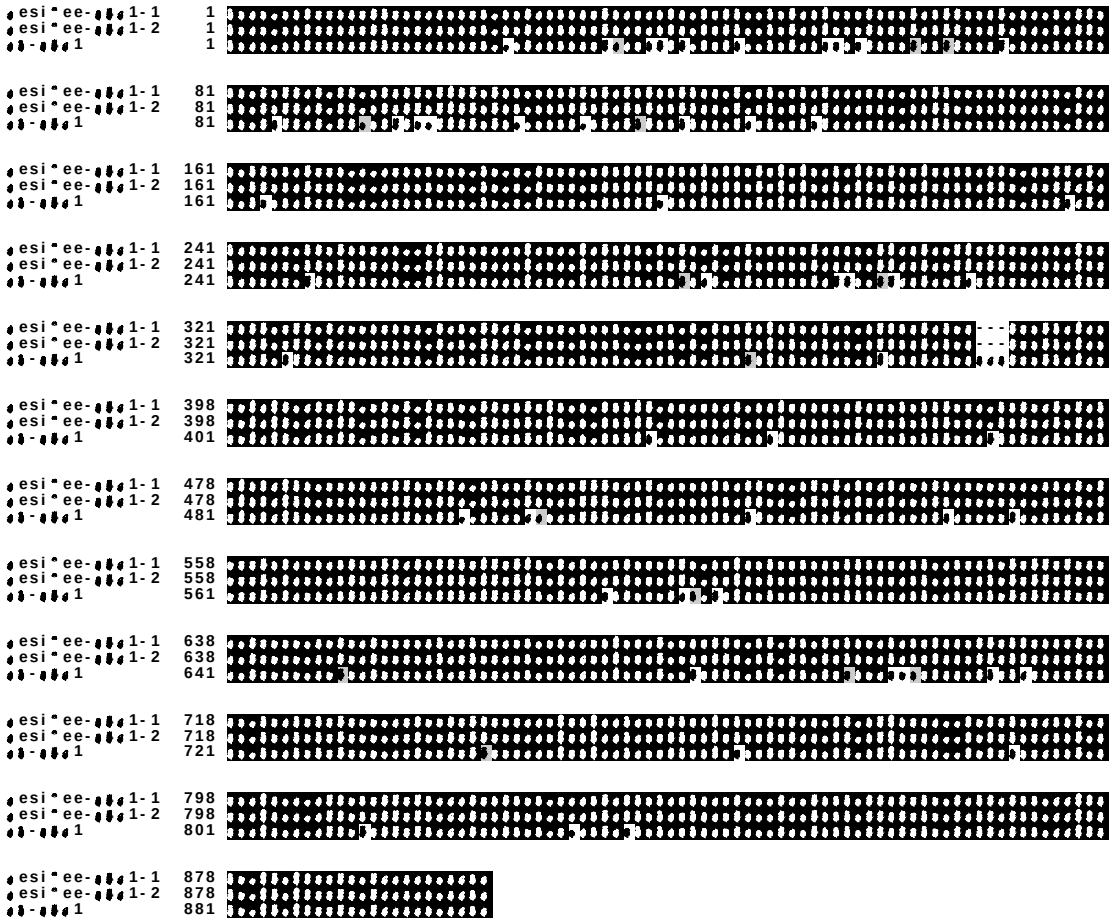


Figure 6: Protein alignment of the NRC1 sequences from Desiree and DM. Protein alignment was done using ClustalW, and analysed with Boxshade.

In contrast with the NRC1 sequences, NRC2 sequences are a lot more conserved. The two NRC2 sequences from Desiree differ only by 2 amino acids from each other (Fig. 7, Table 3), indicating that they could be two alleles of NRC2. Comparing the NRC2 sequences with *S. chacoense,* both have 3 differences; deNRC2.1 on position 875, 752, 152, and 721; deNRC2.2 on position 93, 752 and 152 (Fig. 4, Table 3). Of these differences, the impact of the differences on position 875 is the most profound, as cysteine is hydrophobic while arginine is hydrophilic. Not only on protein-level differences are found; on the level of DNA, there are also multiple synonymous differences between the NRC2 sequences (Fig. S5B).

Table 3: Amino-acid transitions in NRC2 protein sequences.

Position	**dmNRC2**	**deNRC2.1**	**deNRC2.2**	***Sc* NRC2**
93	Threonine	Threonine	Serine	Threonine
875	Arginine	Cysteine	Arginine	Arginine
752	Arginine	Histidine	Histidine	Arginine
152	Threonine	Threonine	Threonine	Methionine
721	Valine	Alanine	Alanine	Alanine

dm= DM, de=Desiree and Sc= *Schacoense*

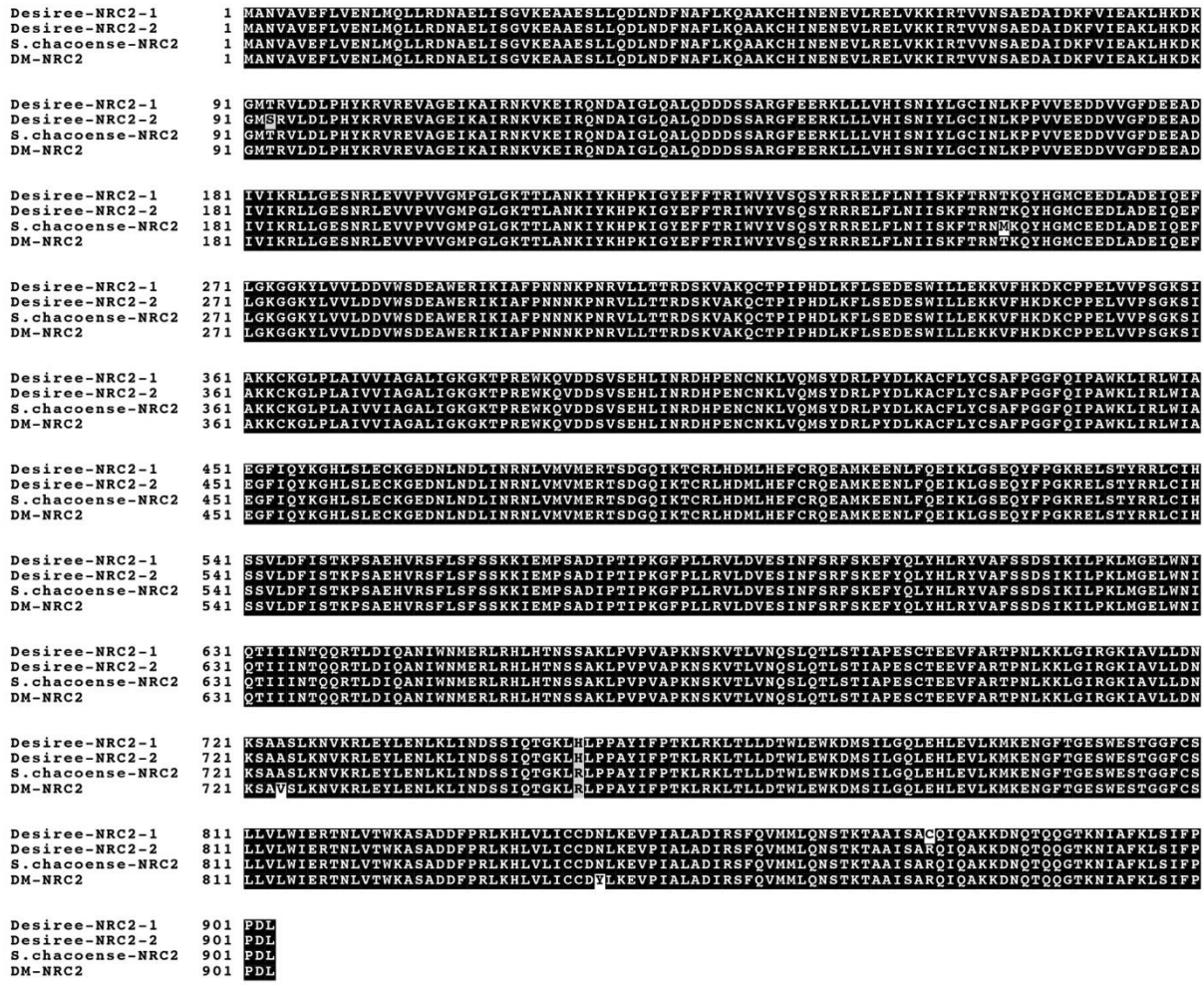


Figure 7: Protein alignment of the NRC2 sequences from DM, Desiree and S. chacoense. Protein alignment was done using ClustalW, and analysed with Boxshade.

Of the new sequences of NRC1 and NRC2, the CDS were determined. These CDS were aligned together with the *in silico* identified NRC sequences from tomato, potato and tobacco. A neighbour-joining tree was produced (Fig. 8). Surprisingly, NRC1 from Desiree does not cluster together with NRC1 from DM. Instead, it clusters more closely together with Sl-NRC1 and with the sequence of St-NRC1a. Based on the small differences between the CDS of NRC2 in Desiree, DM and *S. chacoense*, one allele of NRC2 from Desiree clusters more close together with DM and *S. chacoense* than the other. An alignment of protein sequences yielded the same clustering (Fig. S6).

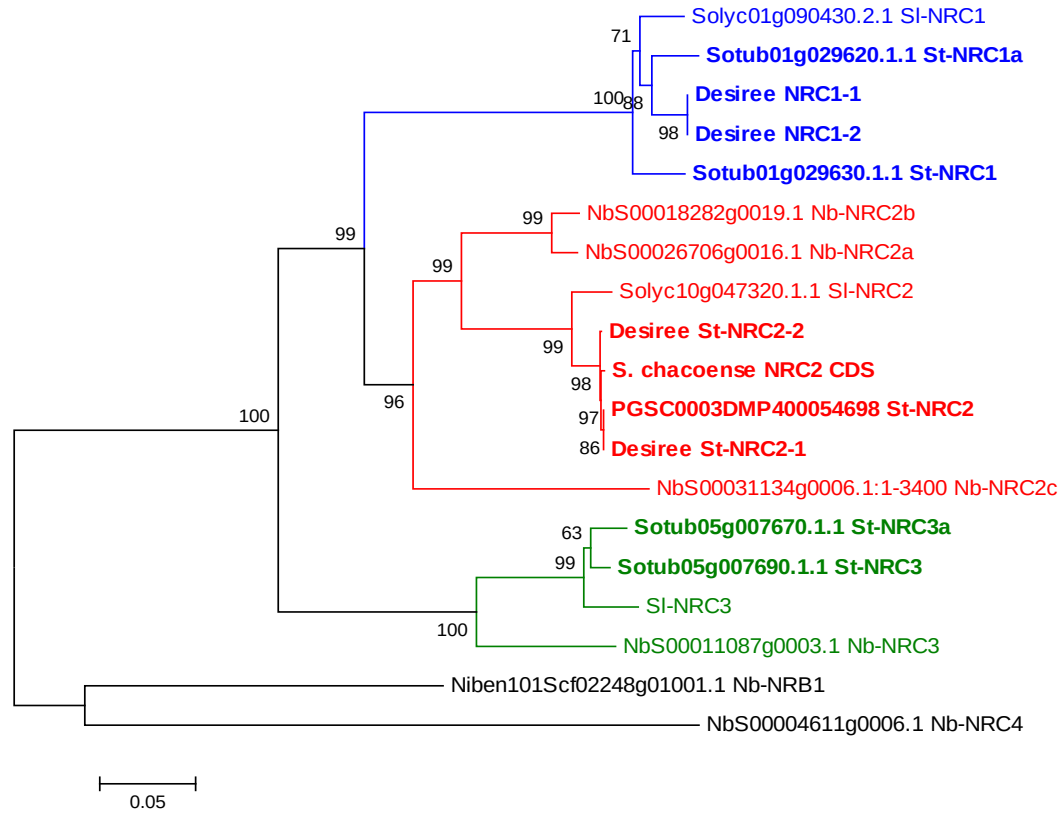


Figure 8. Phylogenetic relationship based on the coding sequences of NRCs and Nb-NRB1 from tomato, potato and *N. benthamiana*, with NRC2 from Desiree and *S. chacoense*. Nb-NRC1 and Nb-NRC4 were not found in the *in silico* allele mining study, but were used in the VIGS experiments described below. Numbers at the branches indicate bootstrap support (1000 replicates). The scale indicates the evolutionary distance in substitutions per position. The NRC1 cluster is coloured blue, NRC2 cluster is coloured red, and the NRC3 cluster is coloured green. Sequences of Nb-NRB1 and Nb-NRC4 were also included. These sequences were not identified in the allele mining study, but Nb-NRC4 was used in the VIGS treatment described below.

Analysis of the cDNA versions of NRC

Because all St-NRCs contain introns, an attempt was done to also make cDNA versions of St-NRCs, so that expression vectors can be made for future agroinfiltrations or VIGS experiments. RNA was harvested at t=0 after infection with *P. infestans*, and cDNA was made. Because RNA of Premiere and DM was not available, RNA of RH89 and 94-2013 was used instead. After the production of cDNA, a PCR was performed to amplify the St-NRC cDNA (Fig. 9, 10). Used primers were a FW primer overlapping the start codon of the CDS in combination with a RV primer overlapping the stop codon of the CDS or a poly-dT RV primer.

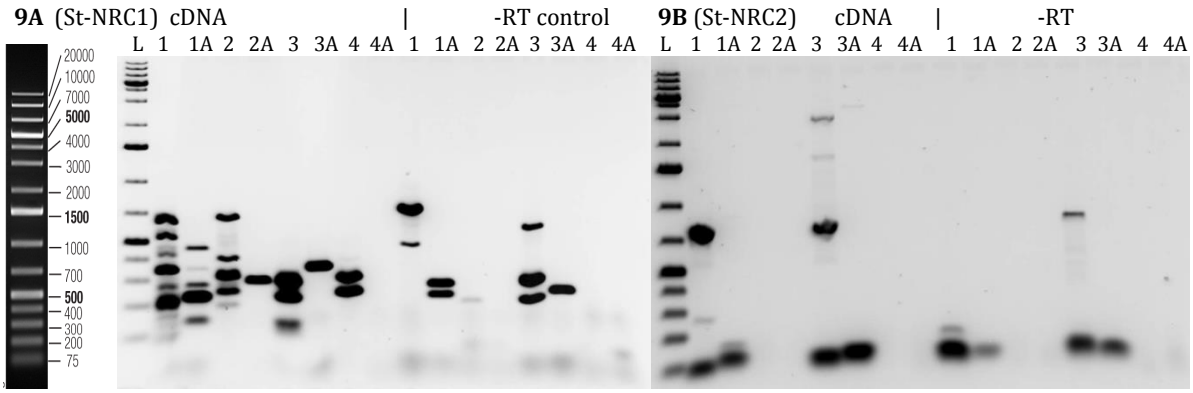
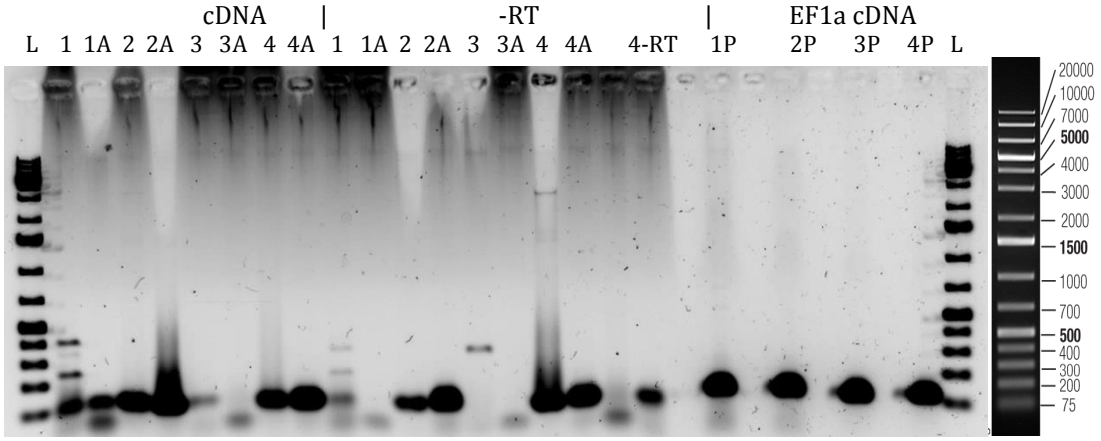


Figure 9: Products of the cDNA on gel. Numbers represent PCR products with primers on the beginning and end of the coding sequence. Numbers with "A" represent PCR products with FW primer on the start of the coding sequence and RV primer on the poly-A tail. 1= 94-2031; 2= *S. chacoense*; 3=Desiree; 4=RH89. Primers were designed to amplify NRC1 (Fig. 9A), NRC2 (Fig. 9B), NRC3a (Fig. 10A) and NRC3 (Fig. 10B).

Several surprising differences were found when primers for NRC1 were used. For all the genotypes, a different banding pattern was produced when a poly-dT primer is used as RV primer (Fig. 9). No bands of the expected size of around 3kb were produced, except for Desiree NRC2 (fig 9b). Each cDNA sample did show bands that do not show up in the –RT control; these bands are therefore cDNA specific bands. When using primers for NRC2, only 94-2031 and Desiree showed cDNA specific bands. For all four genotypes, no bands were produced when using the poly-dT primer as RV primer, except for the primer-dimer bands at 75bp. In this case again, bands were shown that are unique for the cDNA sample.

10A (St-NRC3a)



10B (St-NRC3)

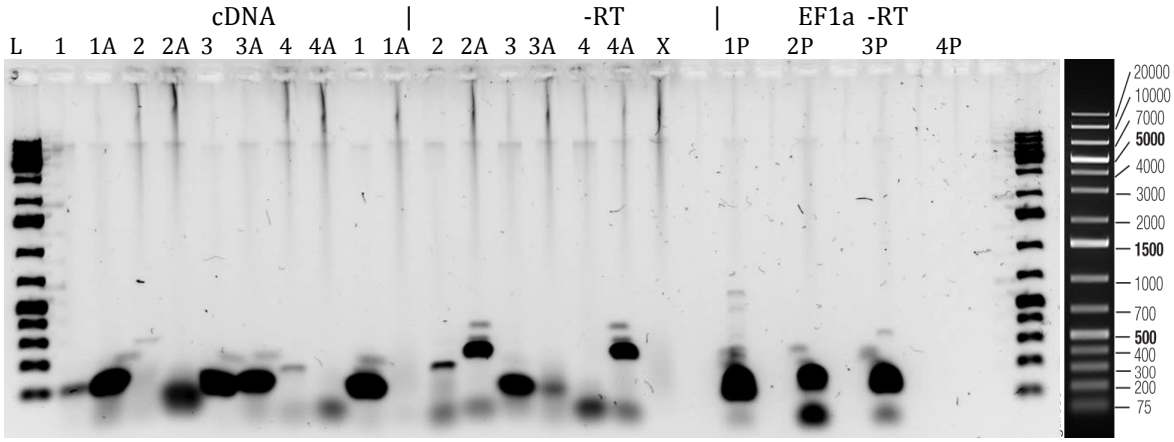
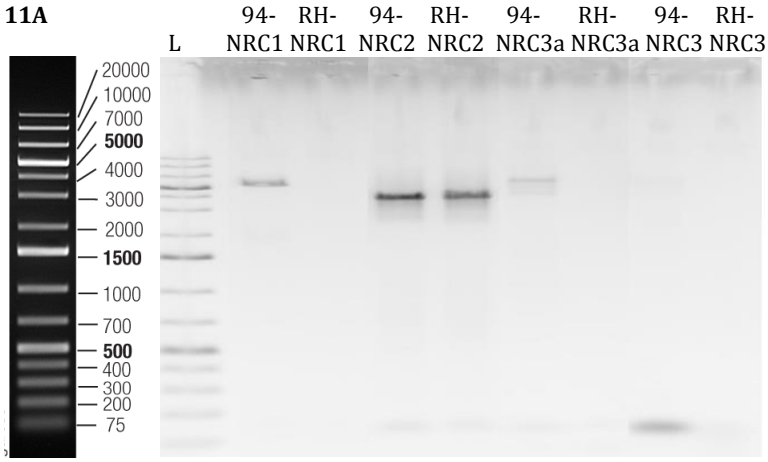


Figure 10: Products of the cDNA of St-NRC3a (Fig. 10A) and St-NRC3 (Fig. 10B) on gel. Numbers represent PCR products with primers on the beginning and end of the coding sequence. Numbers with "A" represent PCR products with FW primer on the start of the coding sequence and RV primer on the poly-A tail. 1=94-2031; 2= *S. chacoense*; 3=Desiree; 4=RH89.

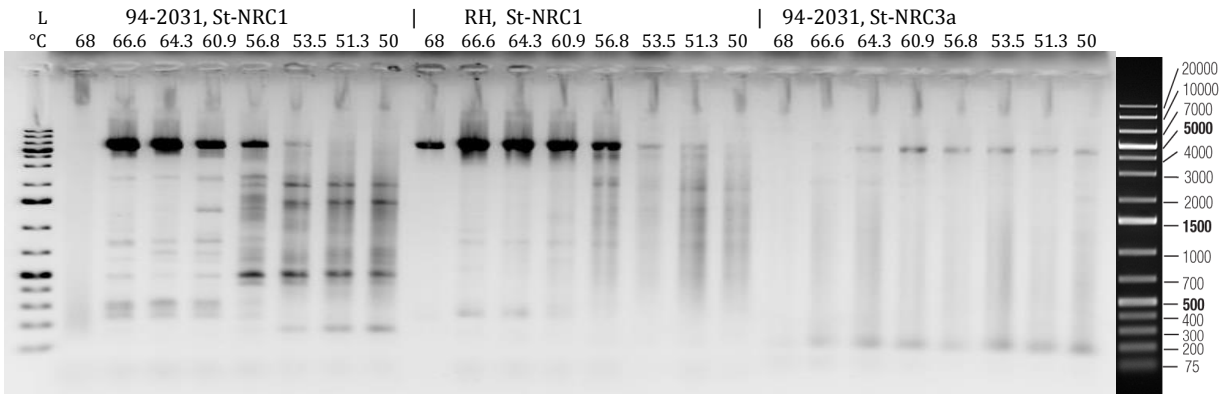
Figure 10A shows the products of the PCR with primers for St-NRC3a. Almost no bands are visible, except for primer dimers, and the bands that are shown are much weaker than in Figure 9A and 9B. For 94-2031, three weak bands are visible that are also visible in the –RT control. For *S. chacoense*, Desiree and RH89, only primer dimers are visible with cDNA. Desiree does show one band in the –RT. As a positive control, EF1a was used, which should produce a band at 125bp. Behind the very strong primer dimer band, a small band is visible between 70 and 200bp in the cDNA; this small band is likely a photographic artefact. In the –RT control, also strong bands were produced for EF1a, including several very weak bands. With 94-2031, two more weak bands between 500 and 700bp were produced. As shown in Figure 10B, St-NRC3 also shows weaker bands compared to the bands produced in Figure 9. In *S. chacoense*, one band at 200bp was produced with normal primers and a band at 300bp with the poly-dT primer both in –RT and in cDNA. For Desiree, both primer combinations yielded a band at 200bp that were unique for the cDNA sample. RH98 shows a band at 200bp with normal primers and no

bands with poly-dT primers that are unique for the cDNA. Interestingly, RH89 also produced a strong band with the poly-dT primer that only occurs in the –RT. To check for the presence of genomic NRC homologs in 94-2031 and RH, a PCR was performed with primers designed on the beginning and end of the NRC coding sequences. As shown in Figure 11A, not all genotype-gene combinations yielded bands on gel electrophoresis. Only NRC2 showed bands in both genotypes. Therefore, a gradient PCR was performed with NRC1, NRC3 and NRC3a (Fig. 11B, 11C). Both in RH and 94-2013, St-NRC3 was not amplified in both PCRs (Fig. 11A, 11C). Furthermore, 94-2031 showed very weak bands of Sotub05g007670 compared to RH (Fig. 11B).

11A



11B



11C

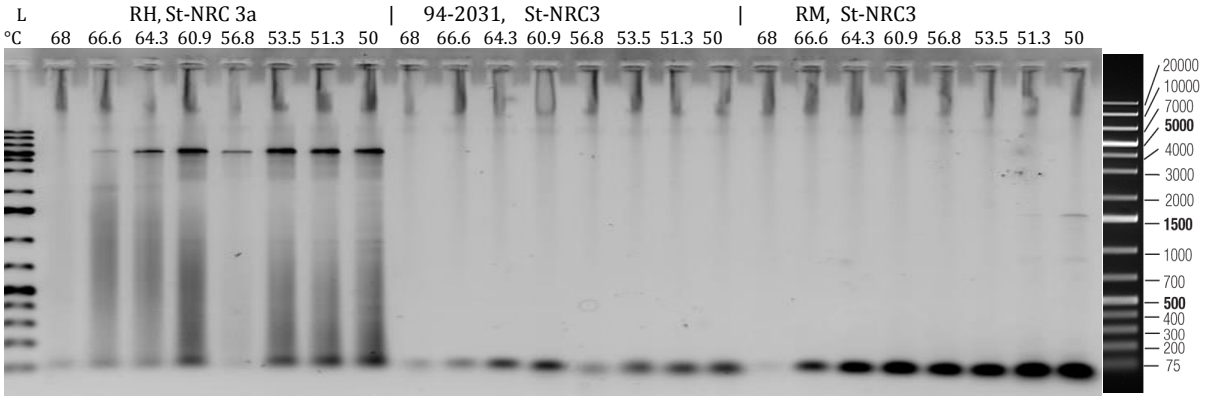


Figure 11: Gel electrophoresis of genomic DNA - PCR products from RH89 and 94-2031. In Figure 11A, 94-NRC represents an NRC from 94-2031, and RH-NRC represents an NRC from RH89.

Virus Induced Gene Silencing of NRCs in *N. benthamiana*

To make an inventory of the Sl-NRC homologs in *N. benthamiana* that are required for the HR triggered by Rpi-chc1, Rpi-chc2, Rpi-ber, Rpi-tar1, R9a and R8, three Virus Induced Gene Silencing (VIGS) experiments were performed (Table 4, Fig. S7, S8, S9). As a control for VIGS, STG1 was used, and as a mock treatment, GUS was used. For each R-gene, a Kruskal-Wallis test was performed to test for significant differences between the VIGS treatments. Overall, a lot of variation within the different treatments was observed, as can be seen in Table 4A and 4B. Within several treatments, some experiments showed a slight increase in HR compared to GUS (Table 4B), while in other treatments, a slight reduction in HR was observed.

Table 4A: HR-response of *N. benthamiana* plants infiltrated 3 weeks after VIGS treatment. Within each row, values that don't share the same letters are significantly different from each other; significance was tested with a Kruskal Wallis test (P<0.05).

VIGS Experiment	Construct	SGT1	SGT1	GUS	Nb-NRC3	NRC2a/b	NRC2c	NRC2a/b +NRC3	NRC4
1	Chc1/Avrchc1	OD=0.3	0.4(a)	1.4(a)	0.9(a)	1.6(a)	1.0(a)	1.2(a)	0.8(a)
2	Chc1/Avrchc1	OD=0.3	0(a)	0.7(b)	0.9(b)	1.2(b)	0.8(b)	0.8(b)	1.2(b)
2	Chc1/Avrchc1	OD=0.6	0(a)	1.2(b)	1.4(b)	1.1(b)	1.0(b)	2.7(c)	1.3(b)
3	Chc1/Avrchc1	OD=0.3	0(a)	1.2(b)	1.2(b)	1.4(b)	1.1(b)	1.0(b)	1.0(b)
4	Chc1/Avrchc1	OD=0.8	0.1(a)	3.5(b)	2.7(b)	2.5(b)	3.6(b)	3.2(b)	2.2(b)
1	Chc2/Avrchc2	OD=0.3	0.6(a)	2.6(b)	1.9(b)	2.3(b)	1.6(b)	2.0(b)	1.8(b)
2	Chc2/Avrchc2	OD=0.3	0(a)	1.1(b)	0.7(b)	1.2(b)	1.0(b)	1.0(b)	2.2(b)
3	Chc2/Avrchc2	OD=0.3	0(a)	1.1(b)	1.5(b)	1.3(b)	1.3(b)	1.8(b)	1.5(b)
3	Chc2/Avrchc2	OD=0.8	0.1(a)	3.7(c)	2.4(bc)	2.0(b)	3.1(bc)	3.1(bc)	1.9(bc)
1	Ber/Avrchc1	OD=0.3	0.2(a)	2.5(b)	1.8(b)	3.7(c)	2.1(b)	3.5(c)	2.8(b)
2	Ber/Avrchc1	OD=0.3	0(a)	2.8(b)	2.1(b)	2.8(b)	3.1(b)	1.8(b)	3.0(b)
3	Ber/Avrchc1	OD=0.3	0(a)	4.0(b)	3.3(b)	3.3(b)	4.1(b)	3.4(b)	2.9(b)
3	Ber/Avrchc1	OD=0.8	0(a)	4.3(c)	3.5(bc)	3.9(bc)	4.4(bc)	4.3(bc)	3.3(b)
1	Tar/Avrchc1	OD=0.3	0.7(a)	2.6(b)	2.8(b)	3.7(b)	2.3(b)	3.4(b)	3.2(b)
2	Tar/Avrchc1	OD=0.3	0.1(a)	2.2(b)	2.4(b)	3.4(b)	2.5(b)	2.7(b)	2.5(b)
3	Tar/Avrchc1	OD=0.3	0(a)	4.9(b)	4.1(b)	4.4(b)	4.0(b)	4.8(b)	3.8(b)
3	Tar/Avrchc1	OD=0.8	0.1(a)	4.8(b)	4.6(b)	4.6(b)	4.7(b)	4.8(b)	4.8(b)
2	Cf9/Avr9	OD=0.3	0(a)	2.4(c)	0.7(b)	1.2(bc)	1.7(bc)	1.3(bc)	1.9(bc)
2	Cf9/Avr9	OD=0.3	0(a)	2.8(b)	1.8(b)	2.2(b)	2.6(b)	1.7(b)	1.8(b)
3	Cf9/Avr9	OD=0.8	0.1(a)	2.9(b)	2.9(b)	2.3(b0	2.8(b)	2.5(b)	2.5(b)
1	R9a/RD28	OD=0.3	1.9(a)	2.6(ab)	2.3(a)	4.0(c)	2.7(ab)	4.0(bc)	3.4(abc)
2	R9a/RD28	OD=0.3	0(a)	2.9(b)	2.0(b)	2.2(b)	2.7(b)	2.0(b)	2.8(b)
3	R9a/RD28	OD=0.3	0(a)	3.6(b)	3.1(b)	2.9(b)	3.7(b)	3.9(b)	2.9(b)
3	R9a/RD28	OD=0.8	0(a)	3.5(b)	3.2(b)	2.7(b)	3.4(b)	3.7(b)	3.1(b)
1	R8/AvrR8	OD=0.3	0.22(a)	2.2(b)	0.9(b)	2.1(b)	1.4(b)	2.2(b)	1.7(b)

Rpi-chc1/AVRchc1 triggered a relatively weak HR response in all three experiments (Table 4A), except when an OD of 0.8 was used. With an OD of 0.8, a slight albeit non-significant reduction in HR compared to GUS was triggered when NRC3, NRC2a/b, or NRC4 was silenced (Table 4B). Still, none of the results from the previous VIGS screen done in 2014 at the Wageningen UR Laboratory of Plant Breeding (Fig. 1) could be reproduced.

Rpi-chc2/AVRchc2 also showed a relatively weak HR response in all treatments including GUS, except with an OD of 0.8. At an OD of 0.8, Rpi-chc2/Avrchc2 showed a significantly reduced HR compared to GUS when NRC2a/b was silenced, and a non-significant reduction in HR when NRC3 and NRC4 was silenced. However, in the first experiment, an increased HR was observed when NRC4 was silenced.

Rpi-ber/Avrchc1 showed a strong HR response in all of the treatments. In the first experiment, silencing of NRC2a/b and silencing of NRC2a/b + NRC3 led to a significantly stronger HR response compared to the other treatments, that could not be reproduced in the repeat experiments. The last experiment also showed a significantly reduced HR response compared to GUS when NRC4 was silenced; also this was not found in the previous experiments. Only silencing of Nb-NRC3 led to a consistent but non-significant reduction in the Rpi-ber triggered HR in all of the experiments.

Infiltration with Tar/Avrchc1 led to a strong HR response in all VIGS treatments, especially in the Last experiment; both at OD=0.3 and OD=0.8. Except for SGT1, no significant reductions in HR were found.

In the first VIGS experiment, R8 was used as a positive control; previous experiments done at the WUR laboratory of plant breeding demonstrated that R8 still triggers HR when NRCs are silenced. Because VIGS of Nb-NRC3 did reduce the R8-triggered HR compared to GUS, Cf9 was used in the repeat experiments. But, unexpectedly, Cf9 did also show a reduced response in all VIGS treatments in the second experiment, although this reduction was not significant in all experiments. VIGS of *NRC3* led to a significant reduction of HR in the second experiment. At a higher OD in the third experiment, this reduction disappeared. Looking at the results of R9a, a big difference can be seen between the first experiment and the repeat experiments. While the first experiment showed an increase in HR when NRC2a/b or NRC2a/b + NRC3 were silenced, this was not found in the repeat experiment. Instead, a slight reduction in HR response was observed when NRC3 or NRC2a/b was silenced in the second and third experiments.
Taken together, a lot of variation was visible between the different experiments and within the treatments in each experiment. Although some significant effects of silencing were observed, none of these significant effects could be reproduced. Still, in plants where NRC3 was silenced, a consistent non-significant reduction of the HR triggered Rpi-Ber was observed. Most of the observed reductions in HR were not very strong; the maximal observed reduction was -1.8 in a HR scale of 0-5. Silencing of SGT1 led to a complete reduction of the HR in all experiments except for R9a in the first experiment. This indicates that the differences within and between the experiments were not due to differences in efficiencies of VIGS.

Table 4B: HR response in NRC-VIGS treated plants compared to GUS-VIGS treated plants. Asterisks indicate significant differences also shown in Table 4A. * Indicates a significant differences compared to GUS, ** indicates a significant difference compared to all all VIGS treatments.

VIGS Experiment	Construct		Nb-NRC3	NRC2a/b	NRC2c	NRC2a/b +NRC3	NRC4
1	Chc1/Avrchc1	OD=0.3	-0.4	0.2	-0.4	-0.1	-0.6
2	Chc1/Avrchc1	OD=0.3	0.2	0.5	0.2	0.1	0.5
2	Chc1/Avrchc1	OD=0.6	0.2	-0.1	-0.2	**1.5****	0.2
3	Chc1/Avrchc1	OD=0.3	0.0	0.2	-0.1	-0.2	-0.2
3	Chc1/Avrchc1	OD=0.8	-0.8	-1.0	0.0	-0.4	-1.3
1	Chc2/Avrchc2	OD=0.3	-0.7	-0.3	-1.1	-0.6	-0.9
2	Chc2/Avrchc2	OD=0.3	-0.4	0.1	-0.1	-0.1	1.1
3	Chc2/Avrchc2	OD=0.3	0.4	0.2	0.2	0.7	0.5
3	Chc2/Avrchc2	OD=0.8	-1.3	**-1.7***	-0.5	-0.6	-1.8
1	Ber/Avrchc1	OD=0.3	-0.7	**1.2***	-0.3	**1.1***	0.4
2	Ber/Avrchc1	OD=0.3	-0.7	0.0	0.3	-1.0	0.2
3	Ber/Avrchc1	OD=0.3	-0.7	-0.7	0.1	-0.6	-1.1
3	Ber/Avrchc1	OD=0.8	-0.8	-0.5	0.0	-0.1	-1.0
1	Tar/Avrchc1	OD=0.3	0.1	1.0	-0.4	0.7	0.5
2	Tar/Avrchc1	OD=0.3	0.2	1.3	0.3	0.5	0.3
3	Tar/Avrchc1	OD=0.3	-0.8	-0.5	-0.9	-0.2	-1.1
3	Tar/Avrchc1	OD=0.8	-0.2	-0.2	-0.1	0.1	0.0
2	Cf9/Avr9	OD=0.3	**-1.7***	-1.3	-0.8	-1.2	-0.5
2	Cf9/Avr9	OD=0.3	-1.1	-0.6	-0.3	-1.2	-1.0
3	Cf9/Avr9	OD=0.8	-0.1	-0.6	-0.1	-0.4	-0.4
1	R9a/RD28	OD=0.3	-0.3	1.4	0.1	1.4	0.8
2	R9a/RD28	OD=0.3	-0.9	-0.8	-0.3	-0.9	-0.1
3	R9a/RD28	OD=0.3	-0.5	-0.7	0.1	0.3	-0.7
3	R9a/RD28	OD=0.8	-0.3	-0.8	-0.1	0.2	-0.4
1	R8/AvrR8	OD=0.3	-1.3	-0.068	-0.75	0.03	-0.51

Overexpression of NRCs in *N. benthamiana*

Previous studies have demonstrated that overexpression of helper NB-LRRs can increase the recognition spectrum and/or the strength of *R* gene triggered HRs. Here, we test whether overexpression of tomato or potato NRCs in *N. benthamiana* can increase the strength of the HR triggered by Rpi-chc1/Avrchc1, Rpi-chc2, Rpi-ber, R8 and R9a. Two experiments were performed, where R/avr, R/avr/NRC combinations were infiltrated in *N. benthamiana* leaves. HR-responses were compared in each leaf. Significant differences were tested using a t-test. In the first experiment, a significant increase of the HR was observed when Rpi-chc1/Avr-chc1 was expressed with Sl-NRC2. This was not reproduced in the repeat-experiment (Fig. 12A, 12B, Table 5). The biggest difference was observed when NRCs were co-expressed with Rpi-ber/Avr-chc1. In both experiments, co-expression of *Sl*-NRC3 or *St*-NRC3 with Rpi-ber/Avr-chc1, significantly reduced the HR (Fig. 12C, 12D, Table 5). For Rpi-chc2, R8 and R9a, no significant differences in the HR were found (Fig. S10).

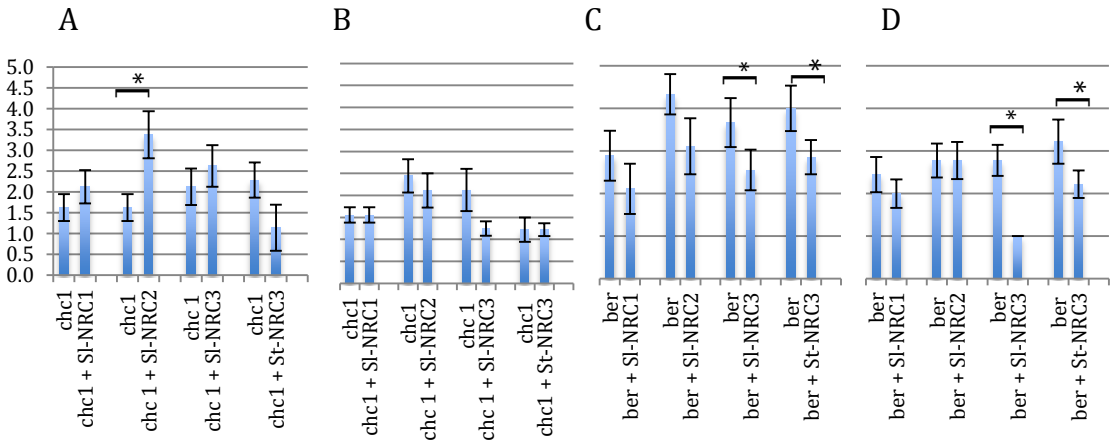
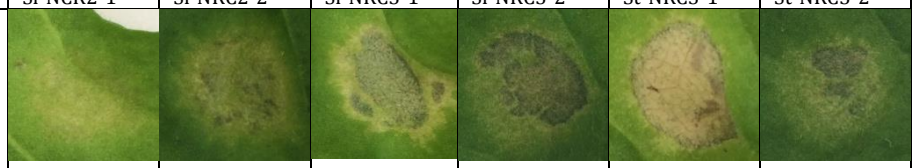
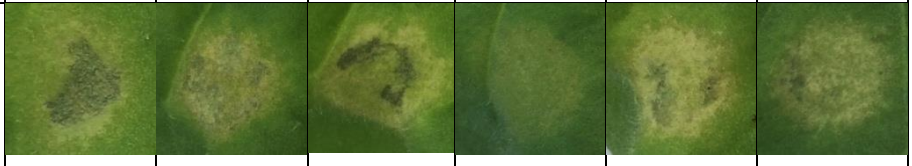


Figure 12: Comparison of HR responses triggered by R/avr and R/avr/NRC combinations. NRCs from tomato (Sl-NRC) and NRCs from potato (St-NRCs) were used. Y-axis represents the HR scale of 0 (no response) to 5 (full necrosis). A t-test test was performed to test for significant differences in each *R* gene treatment. Asterisks represent significant ($p<0.05$) differences. Fig. 12A and 12C represent data from the first experiment; Fig. 12B and 12D represent data from the second experiment.

Table 5: Representative pictures of HR-responses of Chc1 and Ber with and without co-expression of helper NCR. In the table, Sl-NRC2-1 represents pictures from the first experiment with Sl-NRC2, Sl-NRC2-2 the pictures from the second experiment, etc.

	Chc1 Sl-NCR2-1	Chc1 Sl-NRC2-2	Ber Sl-NRC3-1	Ber Sl-NRC3-2	Ber St-NRC3-1	Ber St-NRC3-2
R/avr						
R/avr + NRC						

3. Discussion

In this minor thesis project, by studying helper NB-LRRs, an attempt was made to explain the genetic background-dependent performance of *R* genes. A VIGS screen and Agro infiltration overexpression assay was performed to identify helper NB-LRRs potentially required for potato late blight *R* genes. Also the potato genome was mined to establish the diversity among Sl-NRC1 homologs.

Allele mining and cloning of NRCs in potato

In this study, based on the genome sequence of DM, 5 homologs of Sl-NRC1 were identified (Fig. 1). However, with 2 different primer pairs, Sotub01g029620 could not be amplified with PCR *in vivo.* As the primers were designed on the genome sequence of DM, most likely, this is due to an assembly- or annotation error in the genome sequence. Comparing the diversity of NRCs in tomato, potato and *N. benthamiana*, some interesting differences are seen. *N. benthamiana* has three different NRC2 genes but no NRC1. Tomato has only one version of NRC1, NRC2 and NRC3. Potato on the other hand, has 2 versions of NRC3 and possibly also 2 versions of NRC1, if the presence of St-NTC1a can be validated *in vivo*. All members of the NRC sub clades lie on the same chromosome in all three species (Fig. 3). This indicates that the members of these sub clades may be orthologs. The observation that *N. benthamiana* has three paralogs of NRC2 and potato two paralogs of NRC3 could indicate a different specialization of these NRCs in both plants. In the VIGS-screens done at the Wageningen UR department of Plant Breeding, some slight differences were observed when NRC2a/b and NRC2c were silenced. For example the Prf-triggered hypersensitive response is completely absent when NRC2a is silenced, but only reduced when NRC2c is silenced (Fig. 1). This might thus indicate a diversification of NRC2 proteins in *N. benthamiana,* where different NRC2 proteins are required for different R/AVR genes. In potato, the requirement of NRCs has not yet been tested. Indications for differences in sequences of NRCs among genotypes may have already been observed though. As shown in Figure 11; for 94-2031 and RH, in both PCRs done on genomic DNA, NRC3 could not be amplified. This indicates that these genotypes lack NRC3 or have an allelic variant of NRC3 that is so different that it cannot be amplified with primers designed on the St-NRC3 of DM. Also St-NRC3a shows interesting differences between the genotypes. Of the temperature gradient PCR, RH89 shows strong bands at most temperatures, while 94-2031 shows only very weak bands. Again this might indicate a difference in sequence of St-NRC3a between these two genotypes. Interesting though, the first PCR done at 65C shows a weak band for 94-2031 but no bands for RH. Thus, it must be kept in mind that human error cannot be ruled here. A quantitative PCR with the same temperature gradient can give a good indication of the differences in amplification of NRC3 and NRC3a between the genotypes. Furthermore, obviously, sequencing these NRC3s can give an even better view of the diversity among genotypes.

An attempt was done to sequence the 4 identified NRCs in DM, *S. chacoense*, Desiree and Premiere. Out of the 327 transformed *E. coli* colonies, only 5 gave a complete sequence of a potato NRC. Several explanations can explain this surprisingly low success-rate. First of all, the protocol of the pENTR/dTOPO cloning kit states that when of a PCR product, only single strong bands are visible, non-purified PRC products can be directly used for cloning. As short fragments are far easier to ligate into pENTR/dTOPO, even an on gel (almost) invisible amount of short fragments could ligate in the pENTR/dTOPO vector with an equal or higher frequency than the full-length fragments; this is in correspondence with the high number of sequences that only contained a part of an NRC (Table 2). Another factor explaining the high number of NRC fragments could be the growth speed of *E. coli* cells. As *E. coli* cells with larger fragments grow slower and thus form smaller colonies on plate, they might not have been selected, as they appeared to grow not that well. In retrospect, this explains the unexpected high number of short

sequences found. Many colonies did not show the expected bands at either 300bp or 5kb; many also did not show any bands at all. A possible explanation for the absence of bands might be that the predicted annealing temperatures of the M13 forward (FW) and M13 reverse (RV) primers were too low (48.8 and 46.3, respectively) to yield proper PCR products. The production of aspecific bands is harder to explain, as this can occur for several reasons, such as contamination, sub-optimal primer annealing temperatures, repeat sequences in the DNA sequence, or the absence of primer annealing sites due to the short fragment length.

Of the 5 full sequences that were finally produced, some differences were found between and within the genotypes. Strikingly, the sequences from Desiree and *S. chacoense* were very similar, indicating that NRC2 is very conserved between these two species. The two NRC2 sequences from Desiree were not identical, as they differ both on the level of the protein sequence and the CDS; this indicates that they could be two different alleles. Furthermore, based on the differences in the DNA coding sequence, one of the NRC2s clustered together with a sub cluster of DM and *S. chacoense*, indicating that this NRC2 sequences shares more similarity with DM and *S. chacoense* than with the other NRC2. The high conservedness of these NRC2 genes between cultivated potato and *S. chacoense* does indicate that this gene possibly has an important function, as selection pressure keeps the sequence conserved. In contrast to NRC2, the sequence of NRC1 from DM and Desiree show many differences; in fact, NRC1 from Desiree clusters more closely with Sl-NRC1 than with the NRC1 from DM (Fig. 8). This indicates that NRC1 is not as well conserved as NRC2; at least in the tested genotypes. This, together with the observed potential differences between NRC3 and NRC3a in RH89 and 94-2031 (Fig. 11), demonstrates that there is variation of NRC sequences between genotypes. Thus, it would be very interesting to compare the sequences of all NRC genes and alleles between Desiree and Premiere, and to link these sequence differences with the phenotypic differences observed.

Analysis of the cDNA versions of NRC

For future cloning and sequencing purposes, a cDNA analysis of the 4 NRCs was done in Desiree, *S. chacoense*, RH and 94-2013. Strikingly, when put on gel, almost no fragments of the expected size of around 3kb were observed. Most likely, this is due to the cDNA synthesis kit used. As the kit uses both random primers and oligo(dT) primers, probably only small cDNA fragments were generated, not the entire cDNA. To produce full-length cDNA fragments, instead of incubating at 42C for 30 minutes, 60-90 minutes would have been better, as described in a similar kit where only oligo(dT) primers are used. Still, the results from the cDNA can give an impression of the gene expression of the NRCs. All of the reactions that yielded banding patterns did show a different banding pattern between the PCR with primers on the CDS and FW primer on the CDS and RV primer on the polyA tail. This thus indicates that there might be alternatively spliced transcripts of NRCs in potato. Interestingly, banding patterns of the same genes differed among genotypes. This could be the result of differences in transcript lengths between genotypes. Differing transcripts of NRCs could thus also be an explanation for the background dependence of *R* gene performance. However, it should be noted that this cDNA PCR really needs to be repeated to confirm these findings. First of all, in some cases, no bands at all were visible when RV oligo(dT) primers were used. This could be because these genes are not expressed, but on the other hand human error cannot be ruled out here, as in several of these cases also no primer dimers are visible. Furthermore, as the positive control did not produce high quality bands, this data has to be interpreted with caution. Yet still, a different banding pattern between a CDS primer combination and CDS-poly(dT) combination does give an indication that multiple alternatively spliced transcripts could have been produced. With a cDNA synthesis kit optimized to only produce full-length cDNA, this should be validated by comparing the lengths of different PCR products.

Virus Induced Gene Silencing of NRCs in *N. benthamiana*

Previous experiments done in 2014 at the WUR laboratory of plant breeding demonstrated that VIGS of NRC2a, NRC2c, NRC3 or NRC4 does not reduce the hypersensitive response triggered by Rpi-chc2/Avr-chc2, Cf9/Avr9, R8/Avr8 and R9a/RD28 (Fig. 1). These results could be reproduced, as in this study, also no consistent significant reductions were found. The experiments done in 2014 also demonstrated that silencing of NRC4 leads to a partial reduction of HR induced by Rpi-chc1/Avrchc1. In addition, silencing of NRC2a/2b or NRC2c led to a complete inhibition of the HR triggered by Rpi-chc1/Avrchc1 (Fig. 1). These results thus suggested that NRC2a/b, NRC2c and NRC4 are required for Rpi-chc1/AVRchc1-triggered HR. Surprisingly, these results could not be reproduced in this project. Since silencing of SGT1 did lead to a complete inhibition of HR in all treatments, there is no reason to assume that the VIGS did not work. However, a lot of variation was observed in the treatments within an experiment and between the repeat experiments. In this project, the same plants, same bacteria and same conditions were used as in the experiments done in 2014. Thus, no simple explanation can be given for this big difference. In the first two VIGS experiments, a lower OD was used for agro-infiltration of the *R*-gene/AVR combinations than in 2014 (0.3 vs. 0.5-1.0). Because of this, in first 2 experiments the HR triggered by Chc1/AVRchc1 was very weak; possibly too weak to see reductions caused by silencing NRCs. Still, the last experiment with an OD=0.8 did also not produce results similar to the VIGS screens done in 2014. The only consistent pattern of reduction in HR was found in the HR triggered by Rpi-Ber when Nb-NRC3 was silenced. Although these differences were not found significant, a more thorough statistical analysis such as a split-plot design might characterise these differences as significant. There is thus a slight indication that Rpi-ber requires, at least partially, to trigger a full HR. However, as silencing both NRC2a/b and NRC3 does not lead to any consistent patterns, this conclusion should be drawn with caution. One way to find an explanation for the differences between the previous study and this project could be to repeat the VIGS screen with Rpi-chc1/Avrchc1 with OD values around 0.8, and in addition also measure the gene expression of the NRCs, to check whether NRC2a/2b, NRC2c, and NRC4 are really silenced, as this was not checked in this experiment. For now, it thus remains unclear whether Rpi-chc1/Avrchc1 requires NRC2a/2b or NRC2c, or NRC4 to trigger HR. As no previous experiments were done with Ber/Avrchc1 and Tar/Avrchc1 also here, a repeat experiment with gene-expression analysis should be done to validate the results of this project.

Overexpression of NRCs in *N. benthamiana*

Previous studies have demonstrated that overexpression of helper NB-LRRs can increase the strength of the hypersensitive response triggered by some *R* genes. For example, (Wu et al., 2015b) demonstrated that overexpression of Nb-NRC3 enhances the hypersensitive response triggered by Cf-4 and Pto. In the current study, one experiment showed that overexpression of Sl-NRC2 enhanced the HR triggered by Rpi-chc1 (Fig. 12A), yet this could not be reproduced in the repeat experiment (Fig. 12B). One explanation for this difference is human error. As this experiment was repeated only once, it is not unlikely that something went wrong or was mixed up in either one of the experiments. Thus, another repeat-experiment can most likely clear this up. Interestingly, co-expression of St-NRC3 or Sl-NRC3 with Ber/Avrchc1 led to a reduction in the hypersensitive response in both experiments (Fig. 12C and 12D). Although a third repeat-experiment would be good to solidly confirm these findings, the observation in both experiments that both Sl-NRC3 and St-NRC3 significantly reduced the Rpi-ber-triggered HR already gives a strong first indication that NRC3 has some inhibitory effect on Rpi-ber. One potential explanation for this phenomenon may lie in the competition between NRC proteins in a protein complex. In tomato, Sl-NRC1 is considered to be an important signalling hub for several *R* genes (Gabriëls et al., 2006; Gabriëls et al., 2007; Sueldo, 2014). In *N. benthamiana*, instead of NRC1, Nb-NRC3 has been shown to be the required signalling hub for several *R* genes,

including Pto and possibly also Cf-4 (Wu et al., 2015b). In the VIGS screen performed, a non-significant but consistent reduction in Rpi-Ber triggered HR was observed when Nb-NRC3 was silenced. It is possible that St-NRC3 and Sl-NRC3 are less able to activate defence signalling in *N. benthamiana*. If overexpression of St-NRC3 or Sl-NRC prevents Nb-NRC3 to interact with Rpi-Ber by competing it away from Rpi-ber, this could thus lead to a reduced HR. Another, more speculative explanation for the detrimental effect of overexpressing NRCs may lie in the complex formation of NRC proteins. Previously, it was reported that in tomato, Sl-NRC1 is inhibited by its interaction with one or more other Sl-NRC1 molecules. Upon activation of an R-gene, this oligomer complex is disrupted and thereby activated (Sueldo, 2014). In *N. benthamiana,* Nb-NRC1 does not appear to exist, but instead Nb-NRC3 appears to have a role as signalling hub for several *R* genes (Wu et al., 2015b). Overexpression of *Sl*-NRC3 and *St*-NRC3 might induce the formation of inactive NRC3 oligomer complexes, which are unable or less able to pass on signals downstream.

miRNA, NRCs and R-genes

In this project, the requirement of NRCs for Rpi-chc1 to trigger HR could not be confirmed. Thus, no solid explanation for the differences in performance of Chc1/AVR in and Premiere can be given based on this data. As shown with Rpi-ber, the expression of NRCs can also be disadvantageous for R/genes. This raises the question whether in Premiere, a disadvantageous helper NB-LRR may be present or expressed at a higher level than in Desiree, which causes the lower resistance in Premiere. As was shown in this minor thesis project, not all NRCs show a high conservedness between different potato genotypes. It could thus also be that Premiere has a different allelic variant of one or more St-NRCs. Another explanation for the background dependent performance of Rpi-chc1, which was not tested in this project, could lie with a difference in the activity of miRNAs. In recent years, it has become clear that miRNAs play an important role in resistance in plants, including *Solanaceae*. In 2011, 10 *Solanaceae* miRNA families were identified that target *R* genes (Li et al., 2011). Thus, another possibility could be that in some resistant backgrounds like in Desiree, miRNAs that target *R* genes are suppressed; while in other backgrounds this does not happen. A recent study on tomato demonstrated that these phenomena do occur. In a study by (Zhang et al., 2013), where the susceptible cultivar Moneymaker was compared with the resistant cultivar Motelle, interesting differences in miRNA expression were found. Upon infection with *Fusarium oxysporum,* two *R* gene-targeting miRNAs were suppressed in Motelle but not in Moneymaker. The expression of the target NB-LRRs of these miRNAs increased in Motelle, but not in Moneymaker. Silencing of these targets in Motelle confirmed their role in resistance. Interestingly, none of the target NB-LRRs were resistance genes for *F. oxysporum*. In fact, one of the targets was tm-2*,* the susceptible allele of Tomato Mosaic Virus *R* gene *Tm-2* (Ouyang et al., 2014). This could suggest that *tm-2* is a helper NB-LRR, and that miRNAs can also target helper NB-LRRs. After all, this *tm-2* is not known to be involved in the recognition of *F. oxysporum*, but now appears to be important for the resistance against *F. oxysporum*. However, no evidence for the suppression of NRCs or other helper NB-LRRs by miRNAs in potato has been found yet. A recent study identified and characterised the miRNAs in potato (Zhang et al., 2013). 28 conserved miRNA families were found, with in total 259 distinct miRNAs (Zhang et al., 2013). Target prediction for these miRNAs predicted several resistance genes including *Gpa2*, *R3a*, and *Rpi-blb2*. Furthermore, many unidentified NB-LRRs and LRR-encoding genes, potentially resistance genes, were also predicted as targets. None of the NRCs found in this minor thesis project matches with these miRNA targets (data not shown). Still, all the unidentified MiRNA targets need to be compared with the known Resistance genes in potato. Thus, background dependence of R/AVR function in potato could also, at least in theory, be explained by differential miRNA expression.

Conclusion and recommendations

Overall, the main cause for the background-dependence of the performance of potato *R*-genes remains unclear. Although previous studies at the Wageningen UR Laboratory of Plant Breeding identified NRC2a/b, NRC2c and NRC4 as required helper NB-LRRs for Rpi-chc1, these results could not be reproduced in this study. It would be good to repeat this VIGS screen and also check the gene expression levels of the NRCs to validate these results. As overexpression of NRC3 did reduce the HR response of *Rpi-ber*, the presence of disadvantageous NB-LRRs in non-resistant backgrounds may explain difference in performance of *R* genes in among backgrounds. 4 of the 5 annotated homologs of Sl-NRC1 in potato were confirmed by PCR. The difference in number of NRC2s and NRC3s in *N. benthamiana,* tomato and potato could indicate a different specialisation of NRCs in these species. Out of the sequenced NRCs in this study, no differences were observed that could explain differences in background-dependent success introducing *R*-genes in Desiree and Premiere. However, it was shown not all NRCs show a high conservedness among different genotypes; sequencing the rest of potato NRCs could give a good insight in this.
For future research, it should also be considered to compare the gene expression levels of NRCs among genotypes. Very little is known about the expression levels of helper NB-LRRs, and differences in expression levels between genotypes could also be an explanation of the background-dependence. Results from the cDNA PCR in this study also may indicate the possibility of alternative splicing of NRCs. Comparing banding patterns of cDNA PCR products among genotypes could also give new insights, as some differences were found in banding patterns of the same gene among genotypes in this study. A third possibility that was not studied in this project is the possibility miRNAs that suppress potato R-genes. In recent studies on potato miRNAs, dozens of unidentified resistance genes and NB-LRRs were predicted as targets for miRNAs. Thus, this is also a topic worth exploring.

Materials & Methods

Allele mining and cloning of NRCs in potato

To identify homologs of Sl-NRC1 in potato, tomato and *N. benthamiana*, predicted protein databases, CDS databases and genomic databases were searched on http://www.solgenomics.com and the Spud DB potato genomics resource (http://solanaceae.plantbiology.msu.edu). For Potato, "Potato ITAG release 1" and "Potato PGSC DM v3.4" was used. For tomato, "ITAG release 2.40" was used, and for *N. benthamiana*, the "Genome v.0.4.4." was used. A BLAST was performed on these databases with the protein sequence of Sl-NRC1. Sequences with more than 50% identity were collected. Using MEGAv6, protein sequences were aligned, and a neighbour-joining tree was created. Bootstrap values were also calculated, based on 1000 replications. Using the collected Sl-NRC1 homologs in potato, primers were developed from the beginning and the end of the CDS DNA sequences. DNA was isolated from leaf material of potato genotypes 94-2031, *S. chacoense*, DM, RH, Premiere and Desiree. DNA was isolated using the RETCH-method.

Table 6: Primers developed on the CDS of St-NRCs. Tm temperatures in bold represent the optimal Tm determined with gradient PCR, temperatures in non-bold represent predicted Tm based on primer sequence.

Gene code and gene name	Primer FW (5'-3')	Tm FW (ºC)	Primer RV (5' – 3')	Tm RV (ºC)
Sotub01g029630.1.1 (St-NRC1)	caccATGGTTGATGTGGGGGTGGA	**68**	TTATGAAATGCAATTGGCTTCGCTG	**68**
Sotub01g029620.1.1 (St-NRC1a)	caccATGGTTGATGTGGTGGTTGAT	56,	CTAAGAAGCTGTCTGTACATCAGAATC	56, 57
Sotub01g029620.1.1 (St-NRC1a) longer primers	caccATGGTTGATGTGGTGGTTGATGTGGT	63,	CTAAGAAGCTGTCTGTACATCAGAATCAGGA	63, 61
PGSC0003DMP400054698 (St-NRC2)	caccATGGCGAACGTAGCAGTAGAATTTC	**66**	TCAGAGATCAGGAGGGAATATGGAAAG	**66**
Sotub05g007670.1.1 (St-NRC3a)	caccATGGCGGATGTAGCAGTAAAGTTTTTA	**60**	TTACAATCCAAGATCATGAGGGACTATAG	**60**
Sotub05g007690.1.1 (St-NRC3)	caccATGGCGGATGTAGCAGTAAAG	**65**	TTACAATCCAAGATCATGAGGGAAT	**65**

Using the developed primers, a temperature gradient PCR with a proofreading polymerase (Phusion® High-Fidelity DNA Polymerase) was performed with genotype DM, to find the best annealing temperatures for the primers (Table 6). All PCRs in this project were performed with the Biorad T100™ Termal Cycler. A second temperature gradient PCR was performed with a longer extension step (2 min, 30 sec vs. 1 min, 30 sec) optimised for 5kb fragments. Subsequently, another PCR with optimized annealing temperatures was performed to amplify the fragments of the NRC homologs from all potato genotypes. As St-NRC1a (Sotub01g029620.1.1) from DM could not be amplified in both gradient PCRs, longer primers were developed (Table 6). Also these did not amplify the gene. Therefore St-NRC1a was not used in further analyses. All PCR products except NRC3 from *S. chacoense* and NRC2 from DM produced a single strong band on gel. Therefore, the PCR product was directly used for cloning into pENTR/dTOPO. In an extra temperature gradient PCR, NRC2 from DM did amplify (Fig. S3). NRC3 from *S. chacoense* was purified from gel, but yielded a very low DNA concentration. A second attempt was done to amplify this gene, but only very weak bands were visible on gel. Due to lack of time it was not further used for cloning and sequencing. Chemically competent *E. coli* cells were transformed with the pENTR vectors. Of each NRC gene from each genotype, 24 colonies were selected. Of Premiere-NRC1, only 6 colonies were found on plate, of Desiree-NRC1 16, and DM-NRC3, 17 were found on plate. Of these, all colonies were selected.

Table 7: Primers used in the colony PCR. M13 FORWARD (FW) and M13 REVERSE (RV) align with the vector backbone. The primers with St-NRC are the RV primers that align with the sequence of the insert St-NRC.

Gene	Sequence (5' – 3')	Orientation
M13 FORWARD (FW)	GTAAAACGACGGCCAG	FW
M13 REVERSE (RV)	CAGGAAACAGCTATGAC	RV
St-NRC1	AGCTTCCTTAAGAAAGGC	RV
St-NRC2	AGCTTGCTTAAGAAAGGC	RV
St-NRC3a	AGCTTGTTTGAGGAAAGC	RV
St-NRC3	AGCTTGTTTGAGGAAAGC	RV

A colony PCR was performed with the selected colonies to test for the presence of correct inserts. Each colony was tested with M13 forward (FW) and M13 reverse (RV) primers, and with M13 forward (FW) primer and a RV primer in the insert sequence (Table 7). Each colony that showed bands on gel was used for DNA isolation. Not all NRCs from all genotypes showed bands on gel. Of these, three colonies were selected for further DNA isolation. DNA was isolated and purified using the QIAprep Spin Miniprep Kit. The purified DNA was used for sequencing. First, all prepped colonies were sequenced with the M13 forward (FW) primer. The colonies that yielded good-quality sequences of more than 500bp were subsequently sequenced with the M13 reverse (RV) primers. Colonies that also yielded good quality sequences from the second sequencing reaction were sequenced completely with primers designed on the CDS of DM. For primer sequences, see Table 9.

Of the identified sequences, the CDS was determined by aligning the sequence with the corresponding predicted CDS from DM of this gene. The identified CDS and protein sequences were used for alignment with the protein and CDS of St-NRCs, Nb-NRCs and Sl-NRCs that were found in the *in silico* allele mining. A phylogenetic analysis was performed to find out how the sequenced amplicons relate to each other and to the St-NRCs, Nb-NRCs and Sl-NRCs that were found *in silico*. Using ClustalW in MEGAv6, both CDS and protein sequences were aligned, and a neighbour-joining tree was created. Bootstrap values were also calculated, based on 1000 replications. Furthermore, sequences were aligned in ClustalW on http://www.genome.jp/tools/clustalw/. This alignment was then further analysed using the Boxshade programme on http://www.ch.embnet.org/software/BOX_form.html

Analysis of the cDNA versions of NRC

For future cloning purposes and to test the predicted CDS of the *in silico* identified St-NRC sequences, cDNA was made. As RNA of Premiere and DM was not available, RNA of RH89 and 94-2013 were used instead. RNA was harvested at t=0 after infection with *P. infestans*. First, a DNAse treatment was performed. Then, using the iScript cDNA synthese kit from Biorad, cDNA was made. With the primers designed on the CDS (Table 6), a PCR was performed. Furthermore, also a poly-dT primer was used as RV primer to test for alternative splicing. As a positive control, EF1A forward and reverse primers were also used. To compare the cDNA with the genomic DNA, of RH89 and 94-2013, a PCR was also done on the genomic DNA, with the same primers.

VIGS screens

For the VIGS screens, *Agrobacterium tumefaciens* containing TRV-RNA1 and TRV-RNA2 constructs from (Wu et al., 2015b) were used to infiltrate *N. benthamiana* plants. The VIGS treatments were performed as described in the protocols "Virus‐induced gene silencing" "Agro-infiltration (ATTA) protocol for compartment 6.19 and 6.21" from the Wageningen UR Laboratory of Plant Breeding. The used constructs are: nrc1-11087 (Nb-NRC3), nrc1-26706 (Nb-NRC2a), nrc1-31134 (Nb-NRC2c), nrb1-04611 (Nb-NRC4) and NRC213 (Nb-NRC2a and Nb-NRC3). As a control for VIGS, STG1 was used, and as a mock treatment, GUS was used. In the first VIGS experiment, plants of 3,5 weeks (23 days) were infiltrated with the VIGS constructs. In the second and third experiment, two week-old plants were used. The amount of plants used can be found in Table 8. An OD of 1.0 was used for all VIGS constructs. Three weeks after the VIGS treatment, systemic leaves of these plants were used for further agro-infiltrations. Of each plant, three leaves were infiltrated.

Table 8: Number of plants used in the three VIGS experiments.

VIGS experiment	STG1	GUS	NRC2a	NRC2c	NRC3	NRC4	NRC2a + NRC3
1	4	5	4	3	3	4	5
2	4	4	4	4	4	4	4
3	5	5	5	5	5	5	4

Transient gene expression in VIGS-treated plants

The *in planta* transient gene expression assays were executed as described in the protocol "Agro-infiltration (ATTA) protocol for compartment 6.19 and 6.21". For the VIGS assay, three weeks after the VIGS treatment, the transient gene expression assay was performed. The constructs used were: Chc1/Avrchc1, Chc2/Avrchc2, Ber/Avrchc1, Tar/Avrchc1, R9a/RD28, R8/Avr8, Cf9/Avrcf9. In the first experiment, an OD of 0.3 was used. Because of the weak response of Chc1/Avrchc1, this construct was expressed with an OD of both 0.3 and 0.6 in the second experiment. In the third experiment, all construct were expressed with an OD of both 0.3 and 0.8. The HR was scored at 5dpi, with a scale of 0 (no HR) to 5 (full necrosis).

Overexpression assays

Four-week-old *N. benthamiana* plants were used for the overexpression assays.

Overexpression was performed by co-expressing Sl-NRC1, Sl-NRC2 and Sl-NRC3 with the different R-genes and AVRs. R-genes, AVRs, Sl-NRCs and R-genes + Sl-NRCs were also expressed individually. Two experiments were done. Chc1, Chc2, Ber, Cf9 and R8 were tested. In each plant, the following combinations were expressed all in one leaf: R/AVR; R/AVR + Sl-NCR; R; R + Sl-NRC; AVR; and Sl-NRC. Thus in each plant, one of the R-genes was tested together with one Sl-NRC. For each treatment, three plants were used. Per plant, three leaves were infiltrated. Constructs were mixed in a 1:1:1 ratio; mixtures with only 1 or 2 constructs (such as R/AVR) were filled up with an expression vector with GUS to get equal concentrations for each construct. In both experiments, for Chc1/Avrchc1, an OD of 0.6 was used, and for the other R-genes an OD of 0.3 was used. HR was scored at 5dpi, with a scale of 0 to 5.

Table 9:
Sequences used for the sequencing of the pENTR vectors containing St-NRC genes. On the vector backbone, M13 forward (FW) and M13 reverse (RV) primers were used. For the insert, primers were designed on the CDS of each NRC gene.

Gene	Primer number/name	Sequence (5' – 3')	Orientation
Vector backbone	M13 forward (FW)	GTAAAACGACGGCCAG	FW
Vector backbone	M13 reverse (RV)	CAGGAAACAGCTATGAC	RV
St-NRC1	1	GCCTTTCTTAAGGAAGCT	FW
St-NRC1	2	CACCACATCATCTTCCTC	RV
St-NRC1	3	GAGGAAGATGATGTGGTG	FW
St-NRC1	4	CAATTTAGCAGACGTGTT	RV
St-NRC1	5	AACACGTCTGCTAAATTG	FW
St-NRC1	6	CTGGGATTTAGCAGTTTG	RV
St-NRC1	7	CAAACTGCTAAATCCCAG	FW
St-NRC1	8	CAGCATGCGATTATTTTAG	RV
St-NRC1	9	CTAAAATAATCGCATGCTG	FW
St-NRC1	10	GTCATAAGGCAAAACATCATAAC	RV
St-NRC2	1	GCCTTTCTTAAGCAAGCT	FW
St-NRC2	2	CAACCACATCATCTTCCTC	RV
St-NRC2	3	GAGGAAGATGATGTGGTTG	FW
St-NRC2	4	GTCGTAAGGTAAACGATCATAA	RV
St-NRC2	5	ATGAGTTATGATCGTTTACCTT	FW
St-NRC2	6	ATGGTTTGGATGTTCCAAAG	RV
St-NRC2	7	CTTTGGAACATCCAAACCAT	FW
St-NRC2	8	ACGTGCAGATATTGCTG	RV
St-NRC2	9	CAGCAATATCTGCACG	FW
St-NRC3a	1	GCTTTCCTCAAACAAGCT	FW
St-NRC3a	2	TGATTTTCTTCACCATTTCT	RV
St-NRC3a	3	AGAAATGGTGAAGAAAATCA	FW
St-NRC3a	4	CCGCATCATCTTCCTC	RV
St-NRC3a	5	GAGGAAGATGATGCGG	FW
St-NRC3a	6	CCGCGATCCATAAACG	RV
St-NRC3a	7	CGTTTATGGATCGCGG	FW
St-NRC3a	8	GCTATAGTTGAAAGTGTTTG	RV
St-NRC3a	9	CAAACACTTTCAACTATAGC	FW
St-NRC3	1	GCTTTCCTCAAACAAGCT	FW
St-NRC3	2	TGATTTTCTTCACCATTTCT	RV
St-NRC3	3	AGAAATGGTGAAGAAAATCA	FW
St-NRC3	4	ACCACATCATCTTCCTC	RV
St-NRC3	5	GAGGAAGATGATGTGGT	FW
St-NRC3	6	CCGCGATCCATAAACG	RV
St-NRC3	7	CGTTTATGGATCGCGG	FW
St-NRC3	8	GCTATAGTTGAAAGTGTTTG	RV
St-NRC3	9	CAAACACTTTCAACTATAGC	FW

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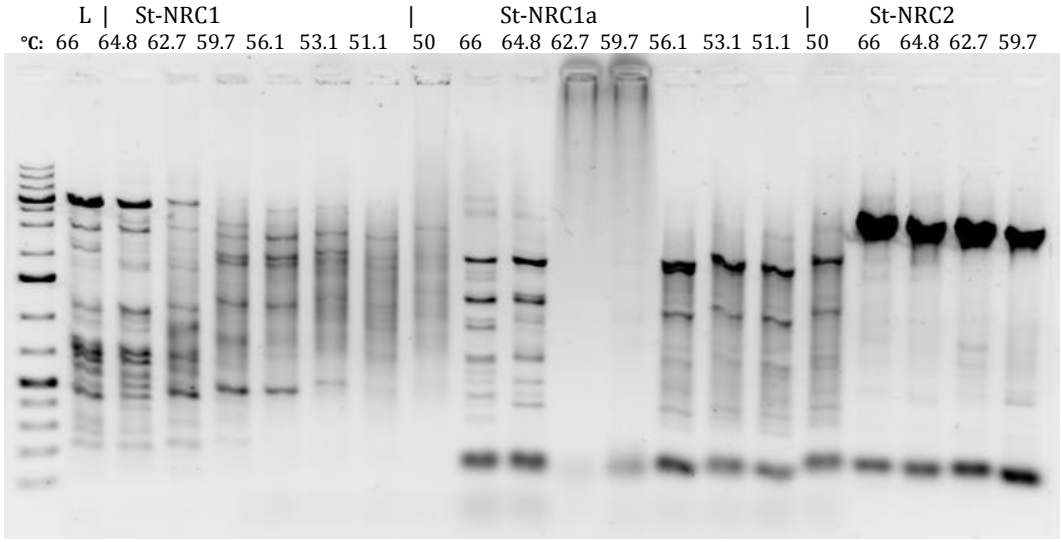
Supplemental Figures

S1A



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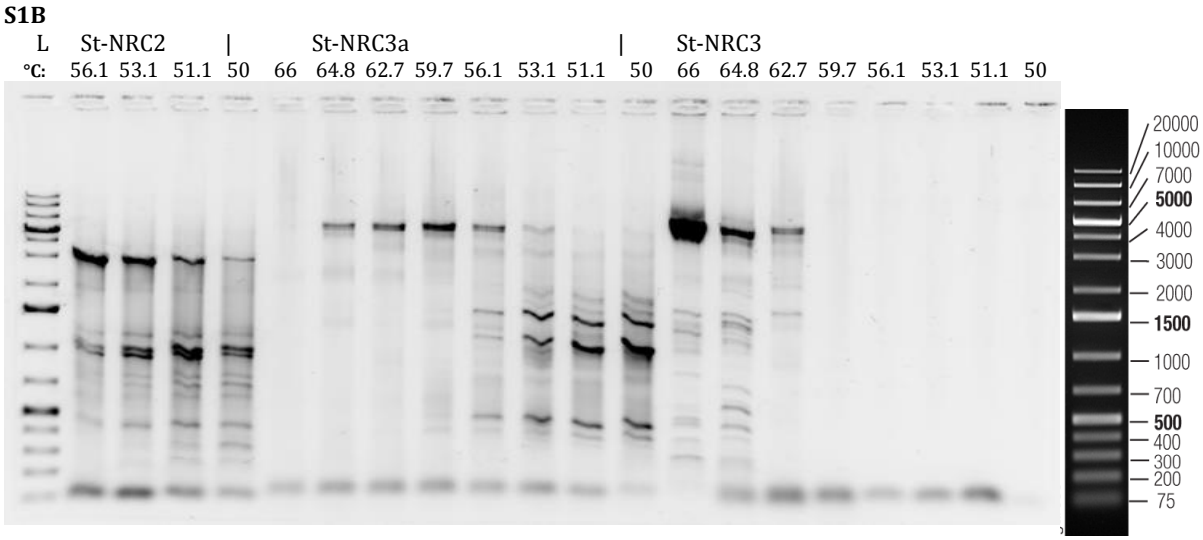

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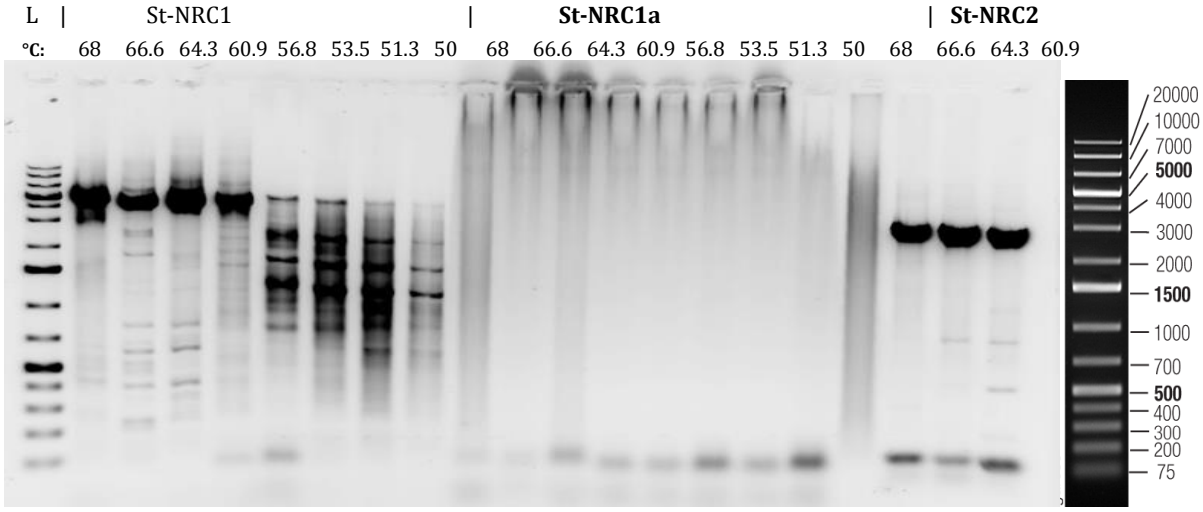

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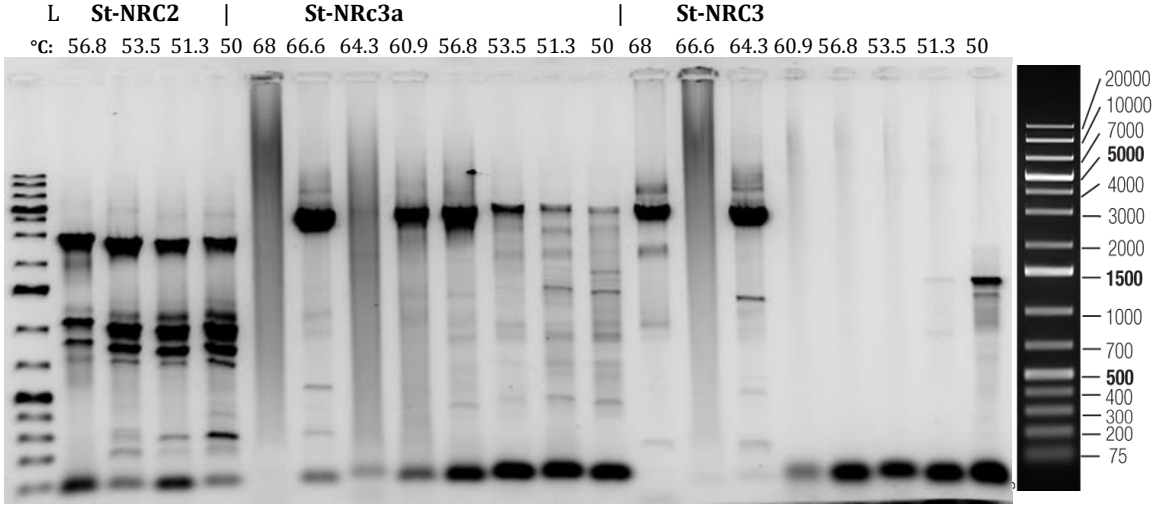


Figure S1: Products of Gradient PCR on gel electrophoresis. Fig. S2A and S2B show products of 66-50C gradient PCR, Fig. S2C and S2D shows products of 68-50C gradient PCR with a longer elongation step in the PCR programme, optimised for 5kb fragments. "L" represents the ladder used.

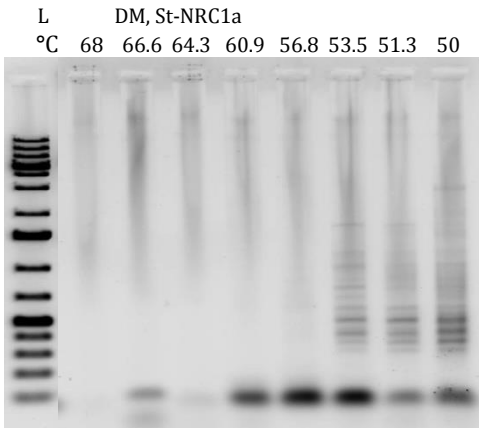


Figure S2: Products of 68-50C gradient PCR with more specific primers for St-NRC1a on DM. "L" represents the ladder that was used.

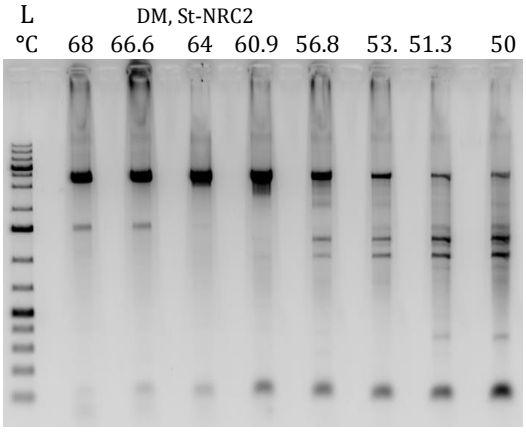


Figure S3: Products of 68-50C gradient PCR with primers for St-NRC2 on DM
"L" represents the ladder that was used.

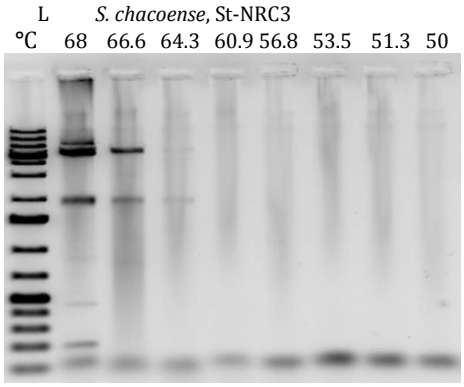
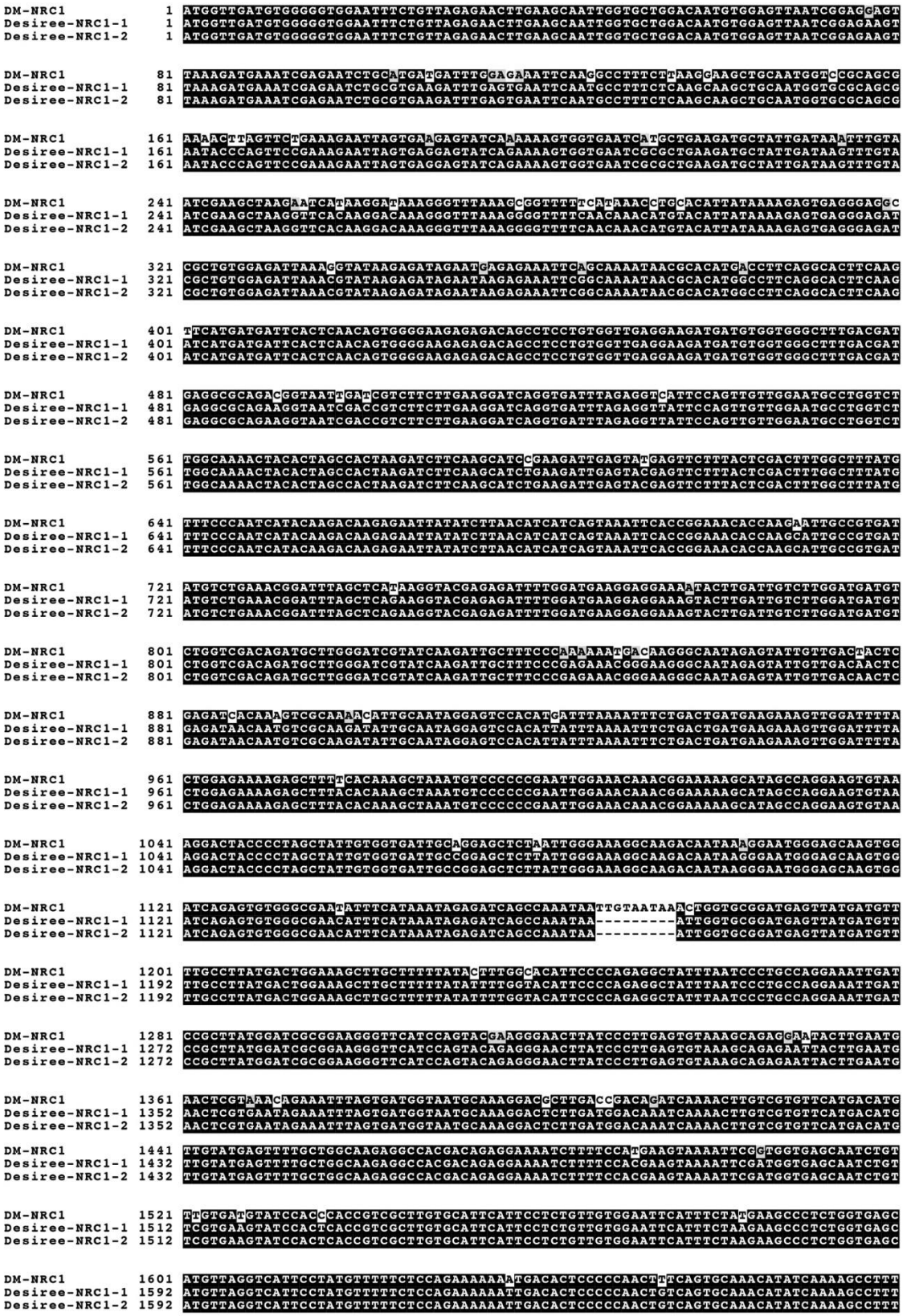


Figure S4: Products of 68-50C gradient PCR with primers for St-NRC3 on *S. chacoense.*
"L" represents the ladder that was used.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Desiree-NRC1-2 1 ATGGTTGATGTGGGGGTGGAATTTCTGTTAGAGAACTTGAAGCAATTGGTGCTGGACAATGTGGAGTTAATCGGAGAAGT
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Desiree-NRC1-1 161 AATACCCAGTTCCGAAAGAATTAGTGAGGAGTATCAGAAAAGTGGTGAATCGCGCTGAAGATGCTATTGATAAGTTTGTA
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Desiree-NRC1-2 961 CTGGAGAAAAGAGCTTTACACAAAGCTAAATGTCCCCCCGAATTGGAAACAAACGGAAAAAGCATAGCCAGGAAGTGTAA
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Desiree-NRC1-1 1041 AGGACTACCCCTAGCTATTGTGGTGATTGCCGGAGCTCTTATTGGGAAAGGCAAGACAATAAGGGAATGGGAGCAAGTGG
Desiree-NRC1-2 1041 AGGACTACCCCTAGCTATTGTGGTGATTGCCGGAGCTCTTATTGGGAAAGGCAAGACAATAAGGGAATGGGAGCAAGTGG
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Desiree-NRC1-1 1272 CCGCTTATGGATCGCGGAAGGGTTCATCCAGTACAGAGGGAACTTATCCCTTGAGTGTAAAGCAGAGAATTACTTGAATG
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Desiree-NRC1-1 1352 AACTCGTGAATAGAAATTTAGTGATGGTAATGCAAAGGACTCTTGATGGACAAATCAAAACTTGTCGTGTTCATGACATG
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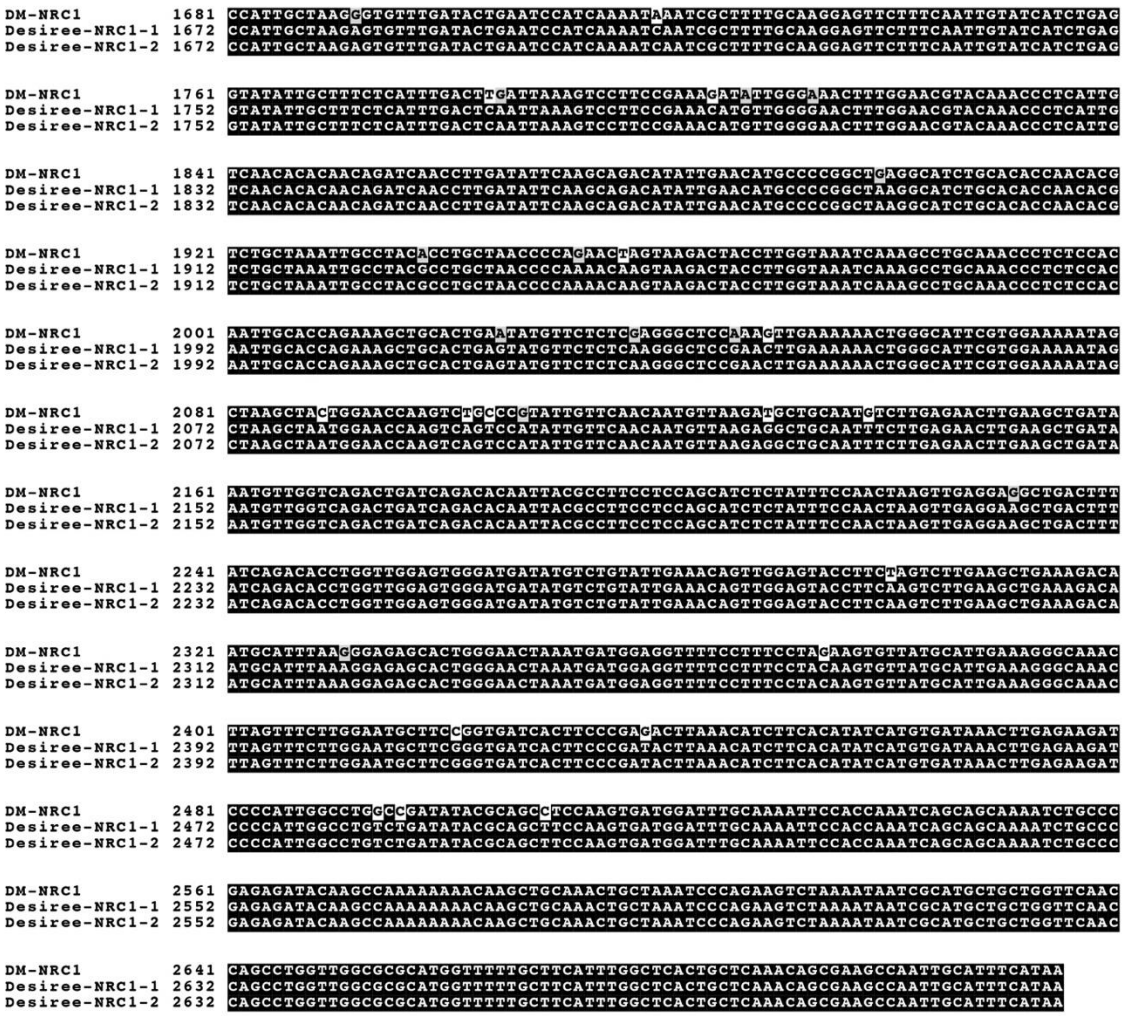
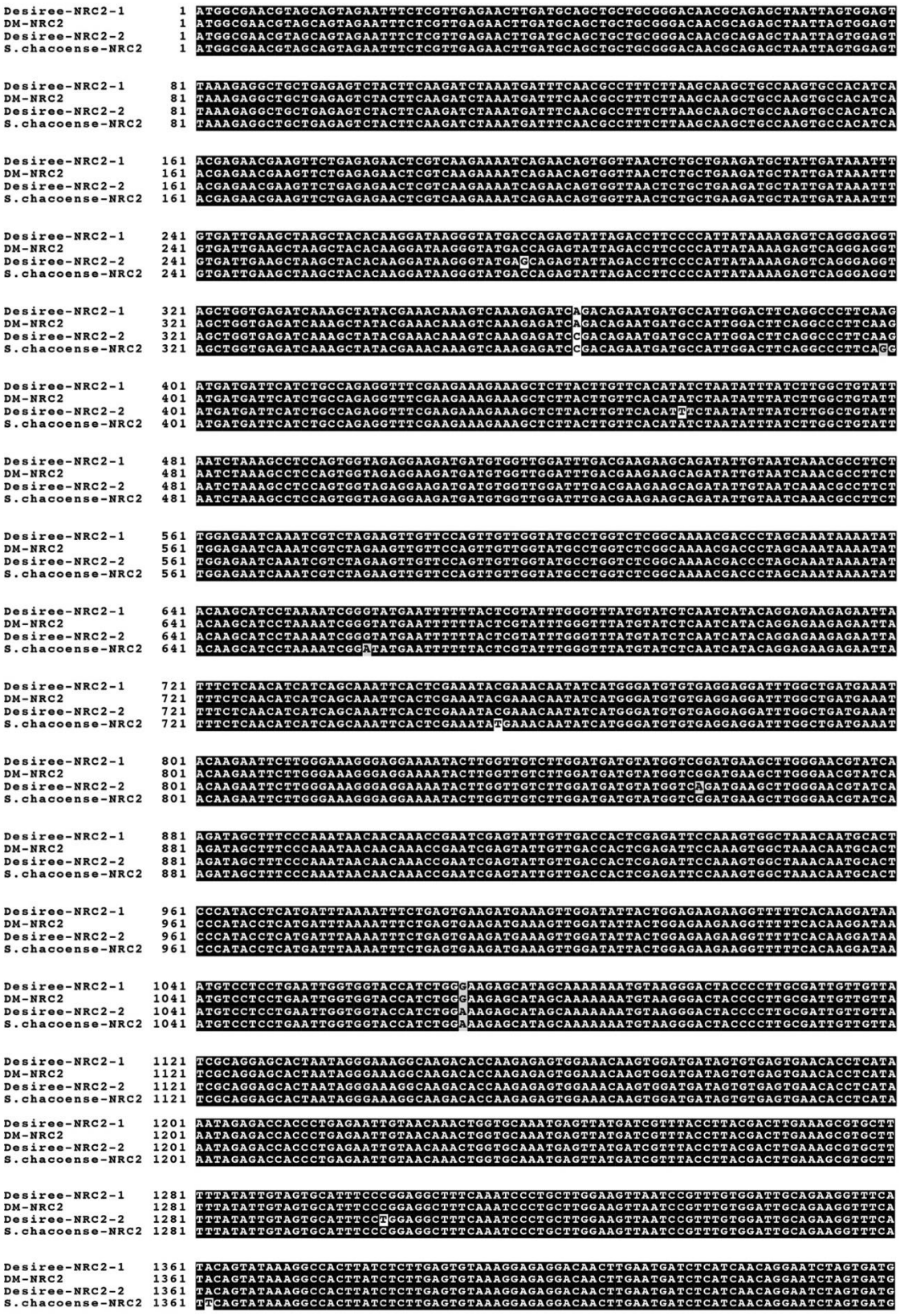


Figure S5A: CDS alignment of the NRC1 sequences from DM and Desiree. CDS alignment was done using ClustalW, and analysed with Boxshade.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Desiree-NRC2-2 81 TAAAGAGGCTGCTGAGAGTCTACTTCAAGATCTAAATGATTTCAACGCCTTTCTTAAGCAAGCTGCCAAGTGCCACATCA
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Desiree-NRC2-2 801 ACAAGAATTCTTGGGAAAGGGAGGAAAATACTTGGTTGTCTTGGATGATGTATGGTCAGATGAAGCTTGGGAACGTATCA
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Desiree-NRC2-2 1361 TACAGTATAAAGGCCACTTATCTCTTGAGTGTAAAGGAGAGGACAACTTGAATGATCTCATCAACAGGAATCTAGTGATG
S.chacoense-NRC2 1361 TTCAGTATAAAGGCCACTTATCTCTTGAGTGTAAAGGAGAGGACAACTTGAATGATCTCATCAACAGGAATCTAGTGATG

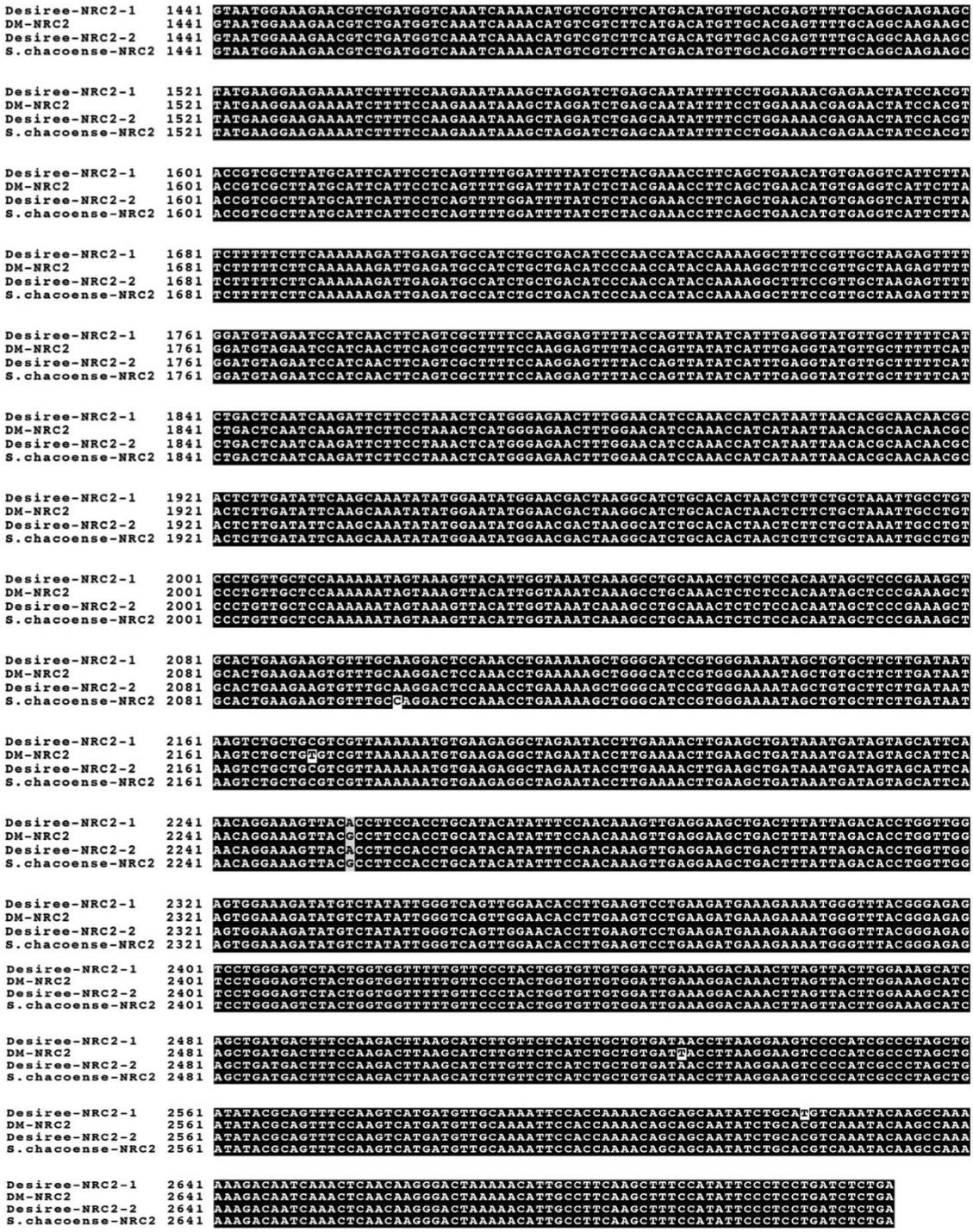

Figure S5B: CDS alignment of the NRC2 sequences from DM, Desiree and S. chacoense. CDS alignment was done using ClustalW, and analysed with Boxshade.

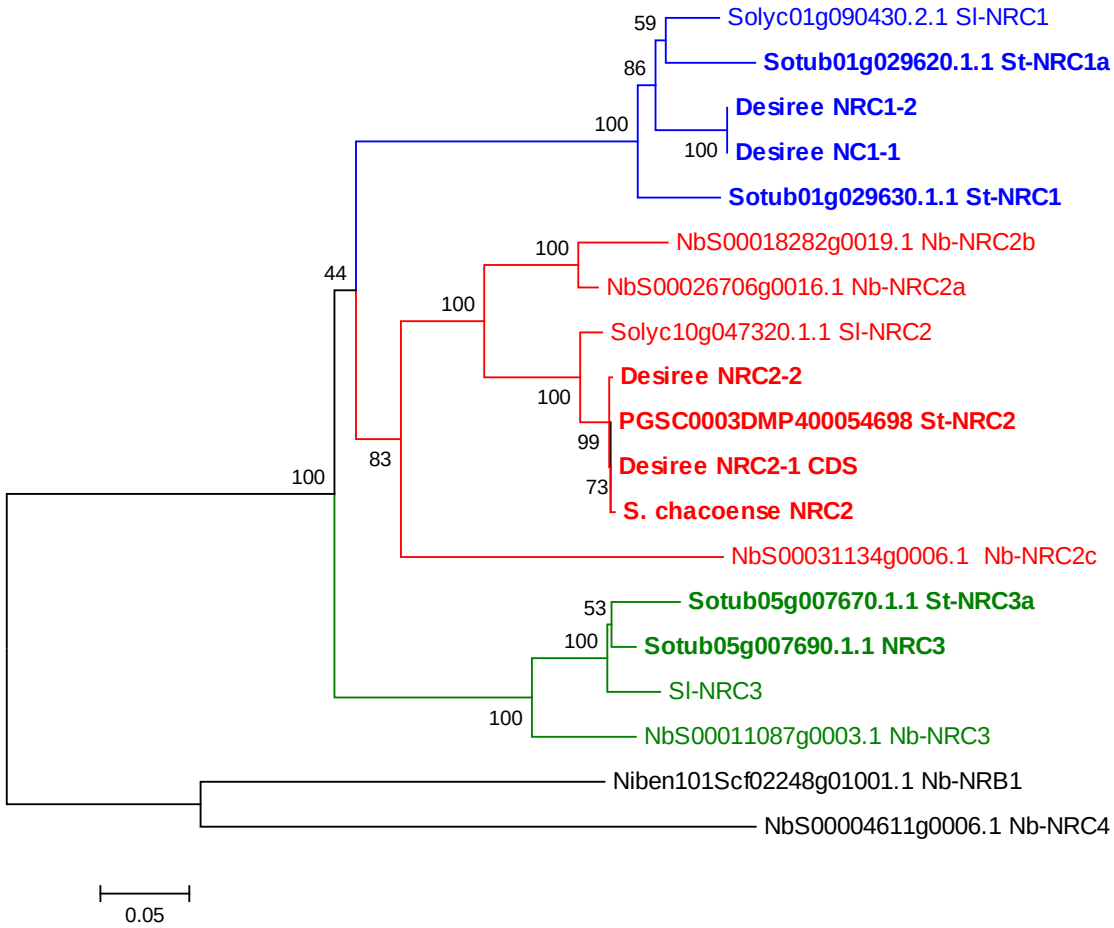

Figure S6. Phylogenetic relationship based on the protein sequences of NRCs and Nb-NRB1 from tomato, potato and *N. benthamiana*, with NRC2 from Desiree and *S. chacoense*. Nb-NRC1 and Nb-NRC4 were not found in the *in silico* allele mining study, but were used in the VIGS experiments. Numbers at the branches indicate bootstrap support (1000 replicates). The scale indicates the evolutionary distance in substitutions per position. The NRC1 cluster is coloured blue, NRC2 cluster is coloured red, and the NRC3 cluster is coloured green. Sequences of Nb-NRB1 and Nb-NRC4 were also included. These sequences were not identified in the allele mining study, but Nb-NRC4 was used in the VIGS treatment described above.

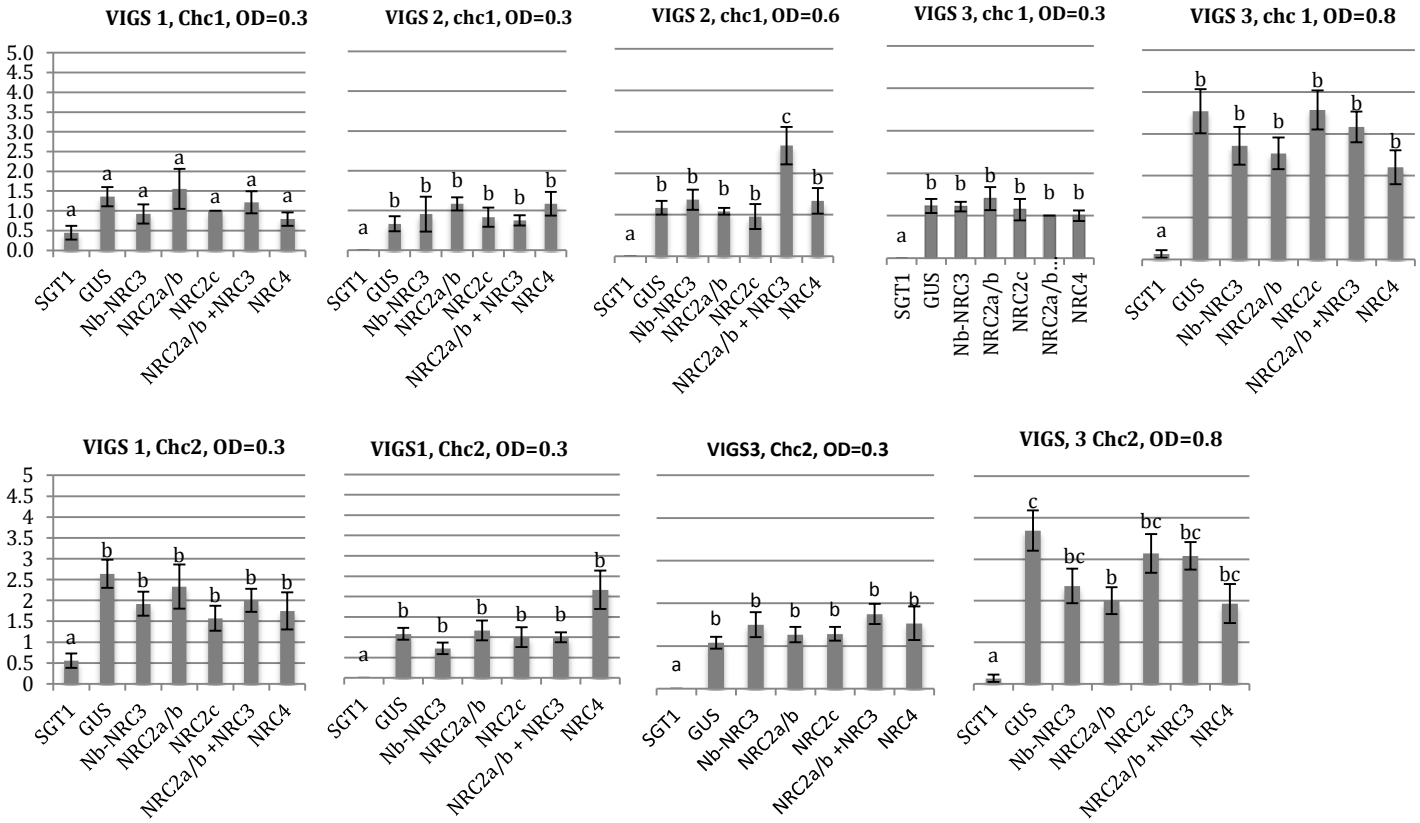

Figure S7: HR responses of VIGS silenced plants. Y-axis shows the HR-scale. HR scale ranges from 0 (no HR) to 5 (full necrosis). X-axis shows the genes silenced with VIGS. For each R-gene, the first graph represents data from the first VIGS experiment, second graph of the repeat experiment. Letters represent significance. Bars that don't share the same letter are significantly different. Significance was determined using a Kruskal Wallis test ($p<0.05$).

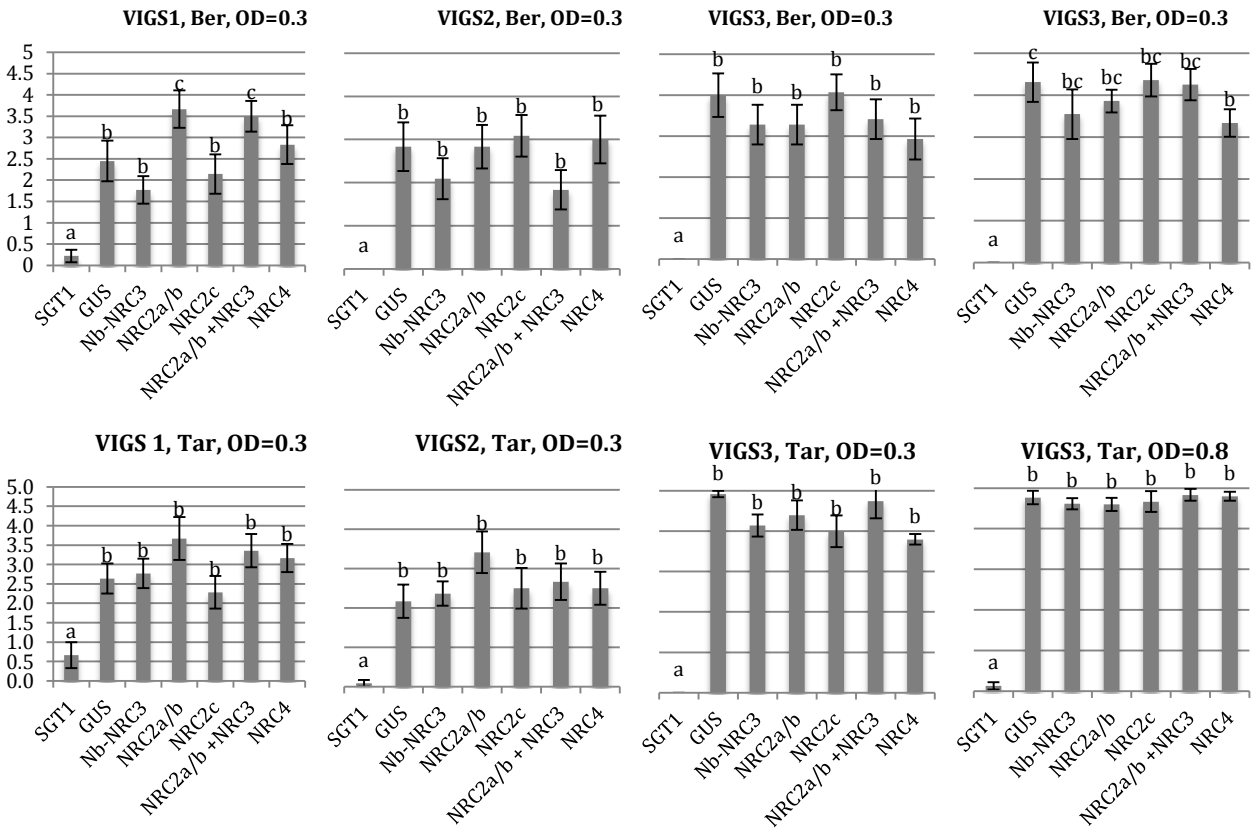

Figure S8: HR responses of VIGS silenced plants. Y-axis shows the HR-scale. HR scale ranges from 0 (no HR) to 5 (full necrosis). X-axis shows the genes silenced with VIGS. For each R-gene, the first graph represents data from the first VIGS experiment, second graph of the repeat experiment. Letters represent significance. Bars that don't share the same letter are significantly different. Significance was determined using a Kruskal Wallis test ($p<0.05$).

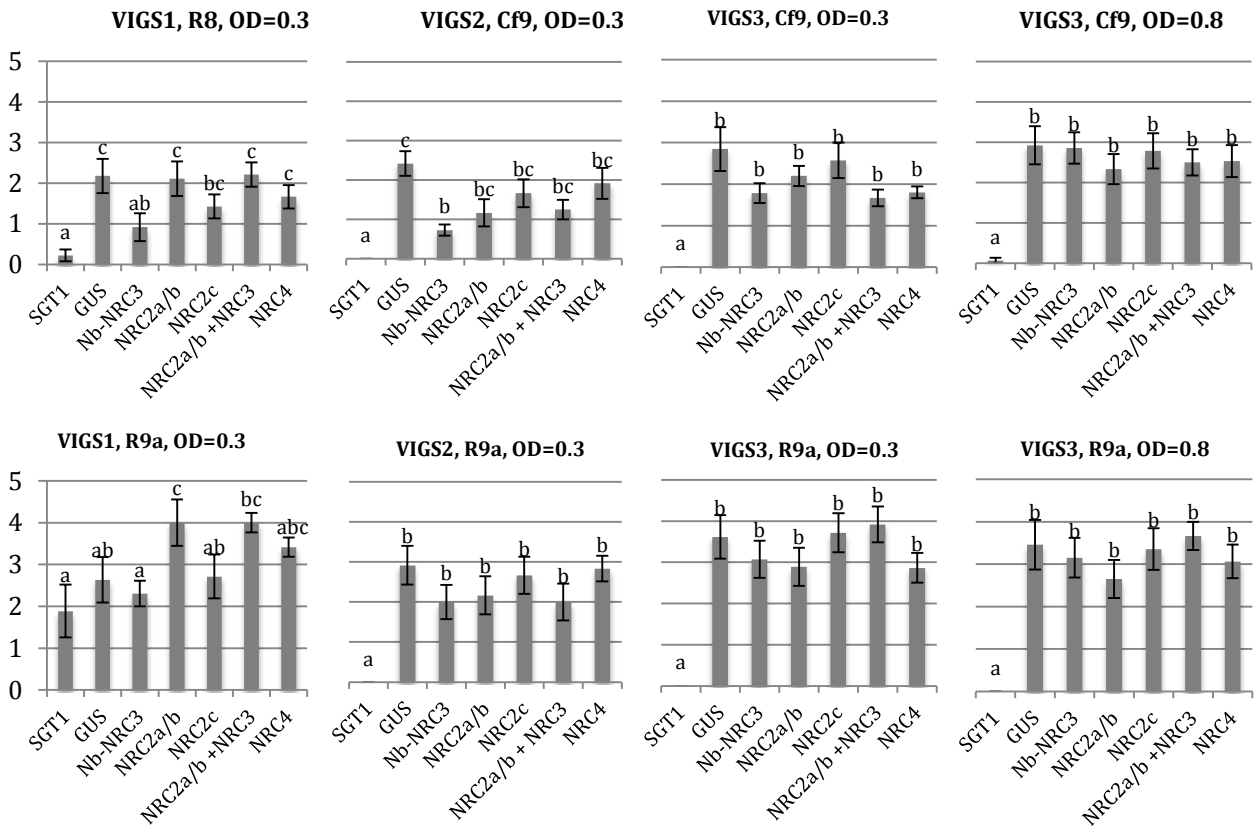

Figure S9: HR responses of VIGS silenced plants. Y-axis shows the HR-scale. HR scale ranges from 0 (no HR) to 5 (full necrosis). X-axis shows the genes silenced with VIGS. For each R-gene, the first graph represents data from the first VIGS experiment, second graph of the repeat experiment. Letters represent significance. Bars that don't share the same letter are significantly different. Significance was determined using a Kruskal Wallis test ($p<0.05$).

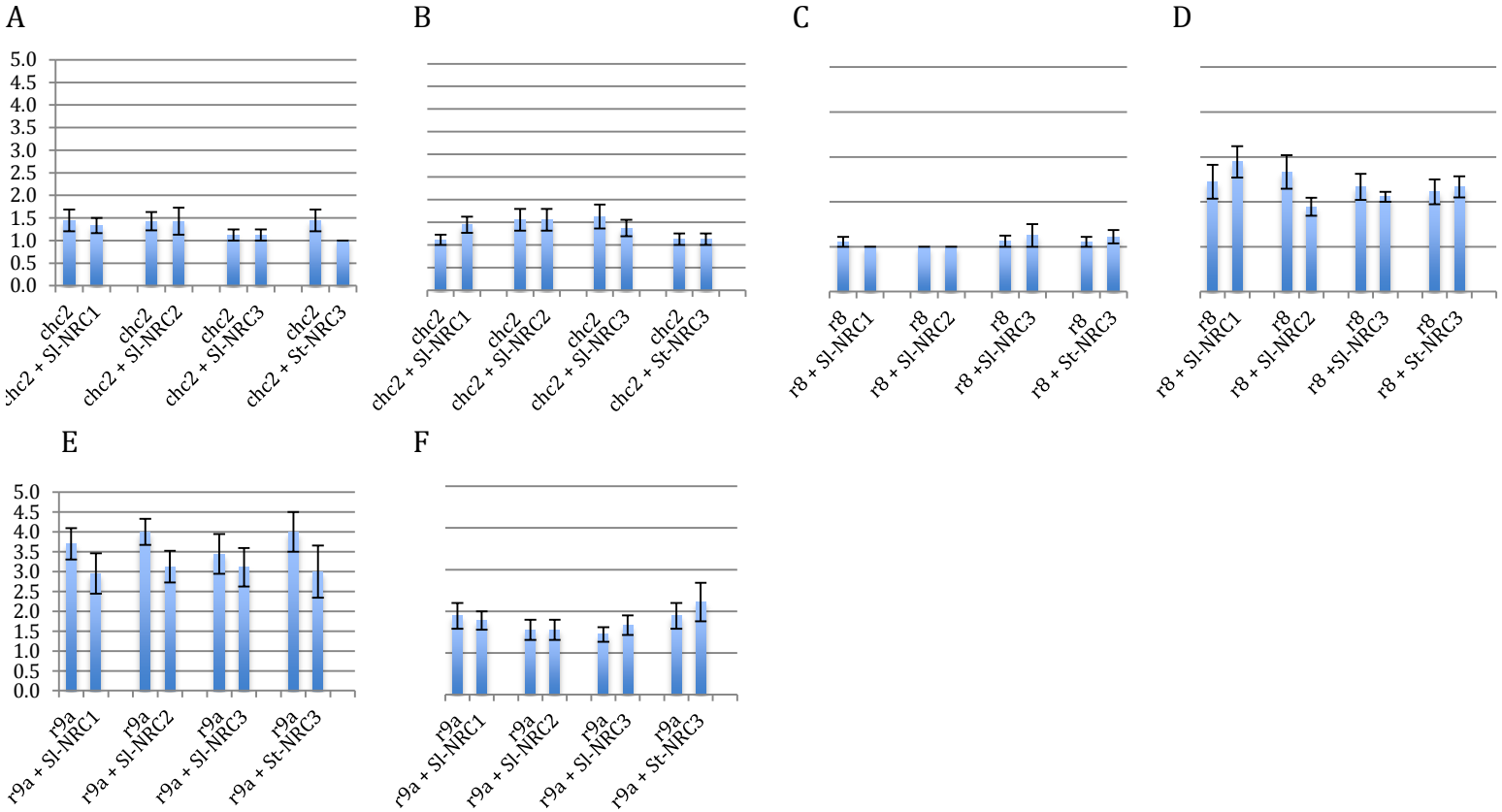

Figure S10: Comparison of HR response triggered by R/avr and R/avr/NRC combinations. Y-axis represents the HR scale of 0 (no response) to 5 (full necrosis). A Kruskal-wallis test was performed for to test for significant differences in each R protein treatment. Asterisks represent significant ($p<0.05$) differences. Big differences were tested for significance with a t-test. Fig. S10A, S10C and S10E represent data from the first experiment; S10B, S10D, and S10F represent data from the repeat experiment.